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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 15/12, 15/00, A01H 5/00

A2

(11) International Publication Number: **WO 98/36084**

(43) International Publication Date: 20 August 1998 (20.08.98)

(21) International Application Number: PCT/US98/02501

(22) International Filing Date: 6 February 1998 (06.02.98)

(30) Priority Data:
08/801,120 14 February 1997 (14.02.97) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ENHANCING PLANT GROWTH USING GENES ENCODING FOR CARBONIC ANHYDRASE, CALCIUM BINDING PROTEIN, METAL BINDING PROTEIN OR BIOMINERALIZATION PROTEIN

(57) Abstract

This invention relates to a process of enhancing plant growth. Growth is enhanced by selecting a plant having at least one heterologous DNA construct with at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein or biomineralization protein, and a plant promoter operatively located upstream of the heterologous gene, and growing the plant in a growth medium. The heterologous gene can be an animal gene of mammalian origin. This invention also relates to a plant comprising the above–mentioned heterologous DNA construct and plant promoter operatively located upstream of the heterologous gene. This invention still further relates to the seeds obtained from these above–mentioned plants.

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TITLE

ENHANCING PLANT GROWTH USING GENES ENCODING FOR CARBONIC ANHYDRASE, CALCIUM BINDING PROTEIN, METAL BINDING PROTEIN OR BIOMINERALIZATION PROTEIN

FIELD OF THE INVENTION

This invention relates to novel methods of enhancing plant growth. The enhanced plant growth is accomplished by selecting a plant having a heterologous DNA construct comprising at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein or biomineralization protein, and any suitable promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium. The heterologous genes include animal, mammalian, bacteria, yeast, plant and synthetic genes.

This invention also relates to plants comprising at least one heterologous DNA construct which comprises at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein or biomineralization protein. A promoter operable in a plant must be operatively located upstream of said heterologous gene insert.

This invention still further relates to the seeds obtained from the above-described plants.

The increased plant growth rates of the present invention can occur in major agricultural and forest crops including monocots, dicots, fruit trees, nut trees, lumber trees, grasses, grains, deciduous trees, coniferous trees and other consumer and industrial crops.

The increase in growth rate varies among different plants and crops and is further dependent upon local growing conditions and the cultivar of the plant. However, plants and crops containing the growth enhancing gene sequences of the present invention will show significant increases in growth rates or in crop yields when compared under identical growing conditions to the same plants or crops not containing these gene sequences. Although these increased crop yields are possible without additional fertilizers, nutrients or water, in some cases the addition of a source of specific nutrients to the soil can augment the effect of these growth enhancing genes.

BACKGROUND OF THE INVENTION

Over the past several decades much effort has been expended on increasing crop yields for a variety of major agricultural and forest products. Some of these methods have included changes in fertilizers, use or application of growth hormones, development of drought resistant varieties, gene insertions for insect and disease resistance, high efficiency watering and nutrient application systems, crop rotation, insertion of genes to extend shelf life and other such efforts. As available agricultural land for crop production

decreases and worldwide populations increase, the demand for methods of increasing crop yields using the same amount of fertilizers, pesticides and water, or even less, will become increasingly more apparent.

To date, research centered on metabolic pathways has only achieved limited success in increasing crop production. What others have found is that a target compound or selected pathway has other limiting steps or factors. Thus, alterations in a targeted pathway have an effect on a related pathway. For example, one approach has been to increase certain plant hormone production levels (such as auxins, gibberellin and cytokinins). However, due to unforeseen limitations or other rate limiting steps, this approach often does not work. As a result, most efforts have concentrated on expression of insect and disease resistance and other genes that have high value in world food markets, such as the FLAVOR SAVOR® delayed ripening genes that result in improved transportation of fruits and vegetables.

Many crops are now reaching their intrinsic limits in terms of the growth rates and sizes that can be achieved using natural selection breeding techniques. At the same time, great strides have been made in various transgenic approaches, such as inserting genes encoding for human growth hormones into fish and similar non-botanical projects, that have the potential for enhancing product yields. However, efforts in plants have not, until the present invention, met with the same type of success.

In contrast, the invention described and claimed in this application relates to the development of a series of gene sequences that, when expressed in major crops, increase growth rates, shorten crop maturity times or

increase crop sizes by affecting one or more of the basic metabolic pathways of photosynthesis.

Photosynthesis involves two separate pathways, called the light and dark reactions. The light reaction is directly dependent on light energy and the dark reaction occurs in the absence of light. In the light reaction, energy is collected by light collection assemblies in the thylakoids. For most of the daylight period, adenosine triphosphate ("ATP") and other energetic compounds accumulate decreasing the rate of the light reaction by feedback inhibition. Once a plant is exposed to light at the start of the light cycle, the light reaction proceeds at full speed. However, for most plants and chloroplasts, the rate of the light reaction is reduced as the level of adenosine monophosphate ("AMP"), adenosine diphosphate ("ADP"), and nicotinamide adenosine dinucleotide ("NADP") decreases with the formation of ATP and $NADPH_2$. This usually occurs within about ten minutes of the start of the light cycle. White, A., Handler, P. and Smith, E.L. (1973) Principals of Biochemistry, McGraw-Hill Book Co., pp. 514-540; Lehninger, A. (1975) Biochemistry, Second Ed. Worth Publishers, Inc. NY, NY. Because of this, researchers have widely believed that the dark reaction must be the limiting reaction of photosynthesis.

Before the work of the present inventors, many people studying photosynthesis assumed that rates could be improved by adding the appropriate enzymes, i.e., enzymes endogenous to the pathways of photosynthesis. In fact, much research has focused on the possibility that the enzyme, ribulose 1,5 diphosphate carboxylase, might be limiting the dark reaction. The supporting evidence included studies showing that diminishing the activity or amount of ribulose 1,5 diphosphate

carboxylase in a plant reduces the overall rate of photosynthesis. While many investigators have sought to increase the concentration of ribulose 1,5 diphosphate carboxylase, there is apparently little proof that this approach is helpful.

Studies of other intermediates involved in the dark reaction also indicate that the ribulose 1,5 diphosphate carboxylase enzyme might be limiting the dark reaction. However, there are some fundamental flaws in the theory that ribulose 1,5 diphosphate carboxylase limits the dark reaction. Carbon dioxide and ribulose diphosphate are the starting materials for making the ribulose 1,5 diphosphate carboxylase enzyme, which comprises 15% of chloroplast protein. These starting materials, however, are present in the environment of the cell in quantities that are in excess of that necessary to make ribulose 1,5 diphosphate carboxylase. For example, because ribulose diphosphate is recycled, it is present in sufficient quantities in the chloroplast and is not rate limiting for photosynthesis. Therefore, the present inventors theorized that the transport of carbon dioxide was the limiting step of the dark reaction.

Carbon dioxide can be transported in a photosynthetic plant cell by both active and passive means. In most plant photosynthetic cells, it would be expected that passive diffusion and convection of carbon dioxide would transport sufficient carbon dioxide to supply the dark reaction at maximum rates. Passive diffusion is defined as the non-enzymatic transport of carbon dioxide into a cell by spontaneous movement from a higher to lower concentration. Convection is defined as the process of a circulating motion of air spaces by the variation in air density.

It was therefore theorized by the present inventors that photosynthetic cells might have greater difficulties in transporting carbon dioxide for any of the following reasons. Because ribulose 1,5 diphosphate carboxylase is thylakoid bound within the chloroplasts, the carbon dioxide would have to travel through the outer cell wall, the cell membrane of the plant cell, the chloroplast membrane, the intracellular air spaces, the cytoplasm of the cell and the cytoplasm of the chloroplast, to reach the site of photosynthesis in a chloroplast thylakoid body. In addition, because a plant leaf typically has a thick cuticle that impedes transport of carbon dioxide from the outside, carbon dioxide mainly enters a leaf through the stoma, which are pores controlled by guard cells that when open allow water loss and gas exchange. Graham et al. disclose at least ten barriers which must be crossed in order to transport carbon dioxide to the active site of ribulose 1,5 diphosphate carboxylase. Graham, D., Reed, M., Patterson, B.D., Hockley, D.G. and Dwyer, M.R. (1984) Chemical Properties Distribution and Physiology of Plant and Algal Carbonic Anhydrases, Annals of the NY Academy of Sciences, Vol. 429:222-237.

As previously mentioned, carbon dioxide must cross the cell membrane to the cytoplasm where it forms bicarbonate. In aquatic plants and algae, it is well known that carbon dioxide is transported via a bicarbonate pathway. $CO_2 + H_2O \rightarrow H_2CO_3$. The present inventors postulated that the reversible exchange of carbon dioxide to bicarbonate may additionally form a necessary and useful storage or transport mechanism for carbon dioxide in land plants as well. If transport and absorptive storage of carbon dioxide are the limiting steps of the dark reaction, then the inventors of the present invention hypothesized that it would be

useful to consider how to maximize carbon dioxide storage and transport rates. The inventors then determined that the effects of carbon dioxide absorptive storage and transport are functions of the enzyme carbonic anhydrase.

A major problem with this approach was that, in plants, native carbonic anhydrase is highly pH dependent and its activity is greatly reduced outside of its optimal pH range. This was a problem because plant carbonic anhydrase gene insertion would mainly be expressed in a pH neutral cytoplasm. To solve this problem, the present inventors considered the possibility of using a naturally occurring form of carbonic anhydrase having higher activity and lower pH dependence. It appeared that carbonic anhydrase from animal origin and, in particular, animal carbonic anhydrase II, might solve the problems of transport and selective absorption of carbon dioxide. Animal carbonic anhydrase is a structural enzyme that is found naturally in animals but not in plants. Therefore, the present inventors hypothesized that carbonic anhydrase from animal origin would increase the photosynthetic rate of plants, and subsequently determined that this was true.

In fact, increased plant growth is achieved in one particular preferred aspect of this invention by using a modified gene sequence encoding for carbonic anhydrase II from an animal source. The carbonic anhydrase enzyme is believed to increase the rate of one of the limiting metabolic pathways of photosynthesis, which involves a catalytic reaction between water and carbon dioxide, by aiding in the transport and absorptive storage of carbon dioxide as carbonate.

The literature and the inventors' experience have shown that most crops, grasses and weeds grow at accelerated rates in the presence of elevated levels of carbon dioxide. It is also known that the earth historically had higher levels of carbon dioxide which might suggest that most modern plants evolved to have maximum photosynthetic rates at only increased carbon dioxide levels. Unfortunately, it is not practical to utilize increased atmospheric carbon dioxide levels to enhance the growth rates of crops grown outside of a greenhouse environment. If, however, as the present inventors theorized, a plant could be genetically made to mimic the effect of increased carbon dioxide levels and therefore force the dark reaction of photosynthesis to proceed at a faster rate, the same increase in growth rate could be achieved. This is one of the objectives of the present invention.

The present inventors also postulated that increased growth rates of plants would occur by providing for the active transport and sinking (or absorptive storage) of limiting nutrients and co-factors that are important to the enzymes of the dark reaction. These include monovalent and divalent cations, such as calcium and zinc, and other nutrients such as phosphorus. The present inventors also theorized that these limiting nutrients might also aid in the transport of carbon dioxide. Divalent cations might also participate in calcium absorption. They subsequently discovered that the lack of calcium uptake in plants limited growth and that these growth limitations seem to be related to calcium pumps that impact the transport of carbon dioxide. They subsequently determined experimentally that the proteins that increased the photosynthetic rate of plants the most were calcium binding proteins, especially those from animal sources, and metal binding proteins, especially the heavy metal binding proteins

such as metallothionein. Inserted in various combinations, the calcium and metal binding genes substantially improve growth rates or shorten the time period required for crop maturity, thus increasing yields.

Initially, the inventors believed that metallothionein would allow for the development of plants that stored heavy metals and that these plants would be useful as mineral supplements and for bioremediation. Plants that acquire heavy metals from the soil and transport them into the plant tissue are said to remediate the contaminated soil. Unexpectedly, even the heavy metal sinking plants also grew faster. In addition, a biomineralization protein, hydroxyapatite nucleating protein, was also tested and unexpectedly found to increase the growth rate of various plants.

Therefore, all four proteins (carbonic anhydrase, calcium binding protein, the metal binding protein metallothionein and the biomineralization protein hydroxyapatite nucleating protein) were found to increase plant growth rates. While the most dramatic increase was observed with carbonic anhydrase II, increased growth also occurred with the separate insertion of the calcium binding protein and the hydroxyapatite nucleating protein genes into plants.

The calcium binding proteins, the hydroxyapatite nucleating protein and the metal binding proteins, along with the carbonic anhydrase enzymes, are all believed to increase the transport and incorporation rate of nutrients and micronutrients, such as calcium, phosphorous and other metals, to the sites of photosynthetic activity of growing tissue. Although each of these genes can function individually, the increased growth rates are best expressed when two or

more of these gene sequences are inserted at the same time.

Transforming the plants of this invention involves inserting single or multiple genes with promoters that are operable in plants. Therefore, one of the objectives of this invention is to insert genes that affect some of the basic metabolic mechanisms of photosynthesis. It is believed that increased growth is due to the ability of carbonic anhydrase to increase the sinking (or absorptive storage) and transport of carbon dioxide. The photosynthesis process is therefore accelerated in much the same way as raising the atmospheric levels of carbon dioxide in a greenhouse. It is also believed that increased growth is due to the ability of the other proteins to increase the rate of absorption of calcium, phosphorus, zinc and other nutrients -- thereby increasing growth rates by supplying calcium for carbon dioxide transport or storage of limiting minerals in the cell in order to maximize the rate of the dark reaction.

In summary, the main objective of the subject invention is to use growth enhancing gene sequences to accelerate metabolic pathways and increase nutrient transport by inserting and expressing them in various crops, to increase the growth rates or yields of plants. The carbonic anhydrase gene and, in particular, the carbonic anhydrase II gene, was found to have the greatest affect on increasing plant growth. The other genes, particularly in combinations, were also found to improve or enhance plant growth.

These and additional objects and advantages of the present invention are shown from the descriptions below.

SUMMARY OF THE INVENTION

This invention relates to a process of enhancing plant growth comprising the steps of selecting a plant having at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein, or biomineralization protein, and a promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium, which includes normal agricultural conditions. In more particular embodiments, the heterologous gene is an animal gene, a mammalian gene, a bacterial gene, a yeast gene, a plant gene or a synthetic gene.

In a further embodiment of the above-described process of enhancing plant growth, the process comprises the steps of selecting a plant having at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one carbonic anhydrase, and a promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium, which includes normal agricultural conditions. In a more particular embodiment, the heterologous gene encoding for the at least one carbonic anhydrase is an animal gene, and in particular, a mammalian gene. In a further embodiment of the carbonic anhydrase process of enhancing plant growth, a source of calcium is added to the growth medium.

In a still further embodiment of the above-described process of enhancing plant growth, the process comprises the steps of selecting a plant having at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene

encoding for at least one calcium binding protein, and a promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium, which includes normal agricultural conditions. The calcium binding proteins consist of a number of diverse proteins that are not necessarily structurally related but which all show a propensity for binding calcium under physiological conditions.

In a more particular embodiment, the calcium binding protein is selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin, calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain, calreticulin, calretinin, calsequestrin, caltractin, gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin. All of the above are groups or classes of calcium binding proteins that are known to people who study calcium absorption or calcium binding proteins and therefore further description is not provided.

In a still more particular embodiment, the heterologous gene encoding for at least one calcium binding protein is selected from the group consisting of bovine, murine, porcine, human, mouse and rat calcium binding protein.

In a further embodiment of the calcium binding protein process of enhancing plant growth, a source of calcium is added to the growth medium, which includes normal agricultural conditions.

In a still further embodiment of the above-described process of enhancing plant growth, the process

comprises the steps of selecting a plant having at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one metal binding protein, and a promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium, which includes normal agricultural conditions.

In a more particular embodiment, the metal binding protein is selected from the group consisting of metallothionein, ubiquitin, zinc binding protein, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5 and HIV-1 enhancer-binding protein.

In a still further embodiment of the above-described process of enhancing plant growth, the process comprises the steps of selecting a plant having at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one biomineralization protein (which is a protein that hyperaccumulates specific minerals), and a promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium, which includes normal agricultural conditions.

In a more particular embodiment, the biomineralization protein is a hydroxyapatite nucleating protein. The word "protein" in hydroxyapatite nucleating protein is used in a broad sense and encompasses peptides which are generally described as amino acid sequences of fifty or less amino acid units. Hydroxyapatite is a defined crystal structure of calcium phosphate only found in living systems. The hydroxyapatite nucleating protein comprises: (i) at least two lysines, which comprise a phosphate binding site, wherein said lysines

are at a distance of 10 Angstroms or less from each other; (ii) at least two aspartic acids, which comprise a calcium ion binding site, wherein said aspartic acids are in a trough; (iii) an alpha helical structure in a portion of the protein; and (iv) a two amino acid distance between the phosphate binding site of (i) and the calcium ion binding site of (ii). In a still more particular embodiment, the hydroxyapatite nucleating protein has at least two phosphate binding sites each containing two lysines at a distance of 10 Angstroms or less from each other. In a further embodiment, the hydroxyapatite nucleating protein comprises the amino acid sequence identified as SEQ ID NO: 1.

In a still further embodiment of the above-described process of enhancing plant growth, the heterologous gene encodes for the hydroxyapatite nucleating protein that comprises the amino acid sequence identified as SEQ ID NO: 1. In a still further embodiment, the heterologous gene encoding for the hydroxyapatite nucleating protein identified as SEQ ID NO: 1 comprises the nucleotide sequence identified as SEQ ID NO: 2.

In a plant embodiment of the subject invention, the plant comprises at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein, or biomineralization protein, and a promoter operatively located upstream of said heterologous gene. In more particular plant embodiments, the heterologous gene is an animal gene, a mammalian gene, a bacterial gene, a yeast gene, a plant gene or a synthetic gene.

The subject plants include, but are not limited to, bean, cabbage, carrot, corn, cotton, eggplant, guayule,

pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato, trees and wheat.

In a still further plant embodiment, the plant comprises at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one carbonic anhydrase, and a promoter operatively located upstream of said heterologous gene. In a more particular plant embodiment, the heterologous gene encoding for the at least one carbonic anhydrase is an animal gene and, in particular, a mammalian gene or a synthetic gene.

In a still further plant embodiment, the plant comprises at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one calcium binding protein, and a promoter operatively located upstream of said heterologous gene. In a more particular plant embodiment, the calcium binding protein is selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin, calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain, calreticulin, calretinin, calsequestrin, caltractin, gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin. In a still more particular plant embodiment, the heterologous gene encoding for at least one calcium binding protein is selected from the group consisting of bovine, porcine, human, mouse and rat calcium binding protein.

In a still further plant embodiment, the plant comprises at least one heterologous DNA construct, the

heterologous DNA construct comprising at least one heterologous gene encoding for at least one metal binding protein, and a promoter operatively located upstream of said heterologous gene. In a more particular plant embodiment, the metal binding protein is selected from the group consisting of metallothionein, ubiquitin, zinc binding protein, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5 and HIV-1 enhancer-binding protein.

In a still further plant embodiment, the plant comprises at least one heterologous DNA construct, the heterologous DNA construct comprising at least one heterologous gene encoding for at least one biomineralization protein, and a promoter operatively located upstream of said heterologous gene. In a more particular plant embodiment, the biomineralization protein is a hydroxyapatite nucleating protein. The hydroxyapatite nucleating protein comprises: (i) at least two lysines, which comprise a phosphate binding site, wherein said lysines are at a distance of 10 Angstroms or less from each other; (ii) at least two aspartic acids, which comprise a calcium ion binding site, wherein said aspartic acids are in a trough; (iii) an alpha helical structure in a portion of the protein; and (iv) a two amino acid distance between the phosphate binding site of (i) and the calcium ion binding site of (ii). In a still more particular plant embodiment, the hydroxyapatite nucleating protein has at least two phosphate binding sites each containing two lysines at a distance of 10 Angstroms or less from each other. In a still further plant embodiment, the hydroxyapatite nucleating protein comprises the amino acid sequence identified as SEQ ID NO: 1.

In a still further plant embodiment, the heterologous gene encodes for the hydroxyapatite nucleating protein that comprises the amino acid sequence identified as SEQ ID NO: 1. In still further preferred plant embodiment, the heterologous gene encoding for the hydroxyapatite nucleating protein identified as SEQ ID NO: 1 comprises the nucleotide sequence identified as SEQ ID NO: 2.

In a seed embodiment, the subject invention relates to the seeds obtained from the above-described plants. These plants include, but are not limited to, bean, cabbage, carrot, corn, cotton, eggplant, guayule, pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato, trees and wheat.

This invention also relates to a process of enhancing calcium accumulation in a plant comprising the steps of: selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one of calcium binding protein, metal binding protein or biomineralization protein, and a plant promoter operatively located upstream of said heterologous gene; and growing said plant in a growth medium. In more particular embodiments, the heterologous gene is an animal gene, a mammalian gene, a bacterial gene, a plant gene or a synthetic gene.

In another embodiment of the process of enhancing calcium accumulation, the heterologous DNA construct comprises at least one heterologous gene encoding for at least one calcium binding protein or peptide. In a more particular embodiment, the calcium binding protein or peptide is selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin,

calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain, calreticulin, calretinin, calsequestrin, caltractin, gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin.

In a further embodiment of the process of enhancing calcium accumulation, the heterologous DNA construct comprises at least one heterologous gene encoding for at least one metal binding protein. In a more particular embodiment, the metal binding protein is selected from the group consisting of metallothionein, ubiquitin, zinc binding protein, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5 and HIV-1 enhancer-binding protein.

In a still further embodiment of the process of enhancing calcium accumulation, the heterologous DNA construct comprises at least one heterologous gene encoding for at least one biomineralization protein. In a more particular embodiment, the biomineralization protein is a hydroxyapatite nucleating protein. The hydroxyapatite nucleating protein comprises: (i) at least two lysines, which comprise a phosphate binding site, wherein said lysines are at a distance of 10 Angstroms or less from each other; (ii) at least two aspartic acids, which comprise a calcium ion binding site, wherein said aspartic acids are in a trough; (iii) an alpha helical structure in a portion of the protein; and (iv) a two amino acid distance between the phosphate binding site of (i) and the calcium ion binding site of (ii). In a more particular embodiment, the hydroxyapatite nucleating protein has at least two phosphate binding sites each containing two lysines at a distance of 10 Angstroms or less from each other. In a further embodiment, the hydroxyapatite nucleating

protein comprises the amino acid sequence identified as SEQ ID NO: 1.

In a still further embodiment of the process of enhancing calcium accumulation, the heterologous gene encodes for the hydroxyapatite nucleating protein which comprises the amino acid sequence identified as SEQ ID NO: 1. In a still further embodiment of the process of enhancing calcium accumulation, the heterologous gene encoding for the hydroxyapatite nucleating protein identified as SEQ ID NO: 1 comprises the nucleotide sequence identified as SEQ ID NO: 2.

In the process of enhancing calcium accumulation in plants, the plants can be selected from the group consisting of bean, cabbage, carrot, corn, cotton, eggplant, guayule, pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato, trees and wheat.

In a further embodiment of the process of enhancing calcium accumulation in plants, a source of calcium is added to the growth medium.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 identifies an amino acid sequence for the subject hydroxyapatite nucleating protein used in the examples of this specification.

SEQ ID NO: 2 identifies a nucleotide sequence of the heterologous gene encoding for the hydroxyapatite nucleating protein identified as SEQ ID NO: 1.

SEQ ID NO: 3 identifies an amino acid sequence for the carbonic anhydrase II protein used in the examples of this specification.

SEQ ID NO: 4 identifies a nucleotide sequence for the heterologous gene encoding for the carbonic anhydrase II protein identified as SEQ ID NO: 3.

SEQ ID NO: 5 identifies an amino acid sequence for the calcium binding protein used in the examples of this specification.

SEQ ID NO: 6 identifies a nucleotide sequence for the heterologous gene encoding for the calcium binding protein identified as SEQ ID NO: 5.

SEQ ID NO: 7 identifies an amino acid sequence for the metal binding protein, metallothionein, used in the examples of this specification.

SEQ ID NO: 8 identifies a nucleotide sequence for the heterologous gene encoding for the metal binding protein, metallothionein, identified as SEQ ID NO: 7.

SEQ ID NO: 9 identifies a nucleotide sequence for the carbonic anhydrase II forward primer used in the examples of this specification.

SEQ ID NO: 10 identifies a nucleotide sequence for the carbonic anhydrase II reverse primer used in the examples of this specification.

SEQ ID NO: 11 identifies the nucleotide sequence for the Hind III Sal I cassette used in the examples of this specification.

SEQ ID NO: 12 identifies the nucleotide sequence for the Hind III Hpa I cassette used in the examples of this specification.

SEQ ID NO: 13 identifies the nucleotide sequence for the Hind III Kpn I cassette used in the examples of this specification.

SEQ ID NO: 14 identifies the nucleotide sequence for the EcoRI Sal I cassette used in the examples of this specification.

SEQ ID NO: 15 identifies the nucleotide sequence for the EcoRI Hpa I cassette used in the examples of this specification.

SEQ ID NO: 16 identifies the nucleotide sequence for the EcoRI Knp I cassette used in the examples of this specification.

DETAILED DESCRIPTION OF THE INVENTION

Sources of the Growth Enhancing Gene Sequences

A brief description of the growth enhancing gene sequences used in the examples of this specification, along with their sources and functions, are summarized in Table 1 below. It should be emphasized that the sources of the genes listed in Table 1 refer to the origins of the gene sequences used in the examples and are not intended to be limiting as to the sources of the gene sequences that can be used in the present invention. For example, similar genes that can accomplish the objectives of this invention may be found in other plants, animals, bacteria and from other sources. Furthermore, those practiced in the art can make small variations in these gene sequences and still accomplish the objectives of this invention.

Table 1

Plant Growth Enhancing Genes

Gene Name	Source(s)	Plant Function(s)
carbonic anhydrase II	animal/synth.	increase CO_2 transport in photosynthesis
calcium binding protein	animal/synth.	absorption/transport of calcium and other metals
metal binding protein	synthetic	absorption/transport of micronutrient metals
hydroxyapatite nucleation protein	bacteria/synth.	matrix/absorption of calcium, phosphorus and other metals

As used in Table 1, a synthetic source means that the gene sequence was constructed by ligating together one or more oligonucleotides made with a DNA synthesizer. In the case of the metal binding protein metallothionein, for example, smaller oligonucleotides were synthesized and then ligated together to form a larger gene sequence. Although these may not represent the full gene sequence of the native gene, they nevertheless retain their functionality. In the case of hydroxyapatite nucleating protein, the entire gene sequence was derived in one step and then other sequences were added to assist in promoting the gene function in the plasmid. For each of the gene sequences used with this invention, small changes can be made in the sequence to slightly alter the function, such as the relative absorption rates of different metals when one or more are present in the soil. Slight variations in the gene sequences may also permit optimization of growth rates for a given plant or agricultural crop.

In addition to the functions disclosed in Table 1, the present inventors discovered that the hydroxyapatite nucleating protein gene also functions to increase the stiffness or tensile strength of wood by forming a hydroxyapatite matrix in the intermolecular spaces of the xylem tissues which are composed primarily of cellulose and lignin. This may also reduce the chlorine requirements during paper pulping processing thus reducing pollution and expense.

Each of the genes used in the subject invention is discussed in detail below.

Carbonic Anhydrases

Carbonic anhydrases from plant origin differ significantly from those of animal origin. In humans, carbonic anhydrases have several forms and are comprised of several subunits that have a molecular weight of about 30Kd (Daltons) for each subunit. In flowering plants, dicotyledons with C3 photosynthesis have two carbonic anhydrase isoenzymes having a molecular weight range of 140Kd to 250Kd, comprising 6 to 8 subunits of 26Kd to 34Kd each. Algae, which comprise a very diverse group, have carbonic anhydrase enzymes ranging in size from 30Kd to over 165Kd. Monocotyledons have two carbonic anhydrase isoenzymes having a molecular weights of about 42Kd to 45Kd. In animals, carbonic anhydrase is often functionally responsible for the transport and storage of phosphorus and zinc. In plants, it is responsible for the transport and storage of zinc atoms, typically one zinc atom per plant carbonic anhydrase subunit. Most plants also exhibit different forms of carbonic anhydrase depending on their cellular location. For example, the carbonic anhydrase in the chloroplast is different from that in the cytoplasm. The activity of these carbonic anhydrase enzymes depends upon various factors within the media in which they function, for example, the concentration of metal ions and pH.

In enzyme reaction kinetics, the rate of catalysis varies with the substrate concentration. For an enzyme that obeys Michaelis-Menten kinetics, K_M is the Michaelis constant and is equal to the substrate concentration at which the reaction rate is half of its maximal value. In plants, the value of K_M for ATP is about 300μM. The value of K_M for carbon dioxide in plants may range from 1 to 200μM and in animals, the value of K_M for carbon dioxide ranges from 100 to 500μM

or more. Animal carbonic anhydrases have a higher metabolic activity. One of the unusual properties of carbonic anhydrases from plant origin is that the enzyme activity is highly variable depending on the pH and phosphate concentration in the surrounding media. For example, changes in pH outside of the optimal range of 5 to 7 coupled with differing levels of phosphate decreases the enzyme activity of carbonic anhydrases from plant origin over a factor of 100 or more. In higher animals, the highest level of carbonic anhydrase activity is found in the carbonic anhydrase II form, and the activity of carbonic anhydrase II is significantly increased in the presence of phosphate. Animal carbonic anhydrases are active at a wider range of pHs than carbonic anhydrases from plant origin. This is especially true for carbonic anhydrase II. In animals, there are four different carbonic anhydrases that have differing evolutionary origins. They can also be characterized by their differing physiological functions. Only three of these carbonic anhydrases, which are called carbonic anhydrase I, carbonic anhydrase II and carbonic anhydrase III, are important to the subject invention.

To increase carbon dioxide transport and activity in photosynthesis, either carbonic anhydrase or enzyme efficiencies need to be increased. This can be achieved by using the carbonic anhydrase I and/or II genes. The activities and physiological characteristics of carbonic anhydrase enzymes are described in the following references. Graham, D., Reed, M., Patterson, B,D., Hockley, D.G. and Dwyer, M.R. (1984) Chemical Properties Distribution and Physiology of Plant and Algal Carbonic Anhydrases, Annals of the NY Academy of Sciences, Vol. 429:222-237; Hewett-Emmett, D., Hopkins, P.J., Tashian, R.E. and Czelusniak, J. (1984) Origins and Molecular Evolution

of the Carbonic Anhydrase Isozymes, Annals of the NY Academy of Sciences, Vol. 429:338-358.

To produce a higher concentration of carbonic anhydrase in plants requires using different carbonic anhydrase enzymes from animal sources and adding sequences to permit ligation and insertion into plant vectors. For increased plant growth rates, the best source of carbonic anhydrase is from animal origin. Mammalian carbonic anhydrase II forms are particularly preferred, especially when altered (to provide for changes in codon and terminal sequences and additional restriction sites) and combined with a suitable plant promoter, such as the 35S promoter. The 35S promoter was originally isolated from a cauliflower virus and is utilized by plant genetics experts for constitutive production of proteins in plants The carbonic anhydrase II gene showed expression in all plants tested, including bacteria such as *Escherichia coli* and *Agrobacterium tumafaciens*, containing the intermediate plasmids. The mammalian and animal carbonic anhydrase forms useful within the subject invention have the types of homologies shown in Table 2.

Table 2

Homology Regions for Animal Carbonic Anhydrases*

Type	Gene Homology Regions of Ancestral Genes															
	7	29	61	92	94	96	106	107	117	119	145	192	194	199	202	202
All Animals	Tyr	Ser	Asn	Gln	His	His	Glu	His	Glu	His	Gly	Trp	Tyr	Thr	Pro	Pro
Additional Conserved Regions After 300 Million Years Ago In Warm Blooded Animals																
	69		200		204		206									
Carbonic Anhydrase I	Lys		His		Phe		Ser									
	67		91													
Carbonic Anhydrase II	Asn		Ile													
	69															
Carbonic Anhydrase III	Val															

Hewett-Emmett, D., Hopkins, P.J., Tashian, R.E. and Czelusniak, J. (1984) Origins and Molecular Evolution of the Carbonic Anhydrase Isozymes, Annals of the NY Academy of Sciences, Vol. 429:338-358.

These homologies are important for defining the necessary active site regions for carbonic anhydrase enzymes from animal origin in order to distinguish them from carbonic anhydrases of plant origin. Looking at the conserved regions after 300 million years of evolution as judged by evaluating the change in these enzymes in animals at different rungs on the evolutionary ladder, it becomes evident which areas are necessary for enzyme activity. The three forms of carbonic anhydrase found in warm blooded animals are preferred in this embodiment for their higher specific enzyme activities. These are called carbonic anhydrase I, carbonic anhydrase II and carbonic anhydrase III. Carbonic anhydrase II is most preferred because it has the highest specific activity. The amino acid sequence of carbonic anhydrase II used in the examples of this specification and originally isolated from human red blood cells is identified as SEQ ID NO: 3. SEQ ID NO: 4 identifies the DNA nucleotide sequence encoding for the amino acid sequence of carbonic anhydrase II identified above as SEQ ID NO: 3. This particular gene was obtained from Montgomery, J.C., Venta, P.J., Tashian, R.E. and Hewett-Emmett, D. (1987) Nucleotide Sequence of Human Liver Carbonic Anhydrase II cDNA, Nucleic Acids Research 15(11): 4687.

Calcium Binding Proteins

To grow most crops faster, an increase in a plant's ability to absorb calcium and phosphorus and an increase in the concentration of both calcium and phosphorus in the growth medium are required. The required concentration of each nutrient varies greatly for different plants. Transport and absorption efficiencies of calcium, however, are increased by using a calcium binding protein gene. To obtain a calcium binding protein that would function in a

diversity of crops, a gene sequence coding for a calcium binding protein from a bovine source was used. It was modified by adding terminal sequences and promoters and inserting it into plant vectors using synthetic gene sequences, called cassettes, that permitted direct splicing into plasmid vectors. Some of the classes of calcium binding proteins that have been found or are expected to be effective in enhancing plant growth are disclosed in Table 3. Class I constitutes specific proteins that bind calcium. These are animal proteins useful in transporting calcium across the intestinal mucosa, which aides in transporting calcium into the bloodstream. Class II constitutes all other classes or types of calcium binding proteins.

Table 3

Animal Calcium Binding Protein Types for Sinking Calcium

Class I

Calcium Binding Proteins (animal absorption or transport related)

Class II

Other classes or types:

Aequorin	Annexin	Cadherin
Calbindin	Calcineurin	Calcitonin
Calcium transporting ATPase	Calcyclin	Calcyphosin
Caccyphosin(e)	Caldesmon	Calgizzarin
Calmodulin	Calnexin	Calpain
Calreticulin	Calretinin	Calsequestrin
Caltractin	Gelsolin	Hydroxyapatite
Osteonectin	Osteopontin	S100
Severin	Transcalcin	Troponin
Tubulin	Villin	

The calcium binding proteins consist of a class of animal absorption and transport related proteins. These proteins are found in the intestinal mucosa and are similar regardless of source. Most of the work related to the present invention has been done with this group of proteins although it is well recognized that other proteins may sufficiently perform the calcium sinking functions of the present invention. Other proteins that may perform these calcium sinking functions are characterized in the following references, which are incorporated by reference. Hitchman, A.J. and Harrison, J.E. (1972) Calcium Binding Proteins in the Duodenal Mucosa of the Chick, Rat, Pig, and Human, Canad. J. Biochem. 50:758-765; Hitchman, A.J., Kerr, M.K., and Harrison, J.E. (1973) The Purification of Pig Vitamin D Induced Intestinal Calcium Binding Protein, Arch. Biochem. Biophys. 155:221-222.

Calcium binding proteins are often found in the intestinal mucosa, which is the mucous-lined membrane that absorbs nutrients through the intestine and aids in the sinking (or accumulation) by reversible binding of calcium and transporting it to the blood stream of animals. SEQ ID NO: 5 identifies the amino acid sequence of the synthetic calcium binding protein used in the examples described below. SEQ ID NO: 6 identifies the DNA nucleotide sequence of the gene encoding the amino acid sequence of the synthetic calcium binding protein identified as SEQ ID NO: 5. This DNA sequence is also disclosed in Brodin, P. and Grundstrom, T. (1986) Expression of Bovine Intestinal Calcium Binding Protein from a Synthetic Gene in *Escherichia coli* and Characterization of the Product, Biochem. 25:5371-5377. The sequence used in the examples, however, was modified from the clone cited in

the Brodin et al. reference as the restriction sites were changed as described later in the specification.

The calcium binding proteins are fairly short proteins that basically act by having reversible calcium binding sites. While there do not appear to be published homology charts to characterize the calcium binding proteins, they are characterized in Table 4 using a representative group that can be found in a few DNA and protein data bases. These are intended to be illustrative of the types of calcium binding proteins that can be used with the subject invention. They are not, however, intended to limit the invention in any manner.

Table 4

Animal Calcium Binding Proteins

Data Bank	Accession Code	Brief Description
GeneBank®*	BOVCABP	Bovine vitamin D-Dependent calcium binding protein mRNA
"	CHKCABPL	Chicken calcium-binding protein in macrophages
"	HSMRP14	Human mRNA for calcium-binding protein
"	MMCABIN	Mouse mRNA for calcium-binding protein
"	MMCBPR	Mouse mRNA for placental calcium-binding protein
"	RATCALBIV	Rat intestinal calcium binding protein
"	ATCAKBPM	A. thaliana mRNA for calcium binding protein
"	DDU03413	Dictoyostelium discoideum AX2 calcium binding protein in mRNA
"	EGMCABP	E. granulosus mRNA for calcium binding protein
"	SCCDC31B	S. cerevisiae CD31 gene for calcium-binding protein
"	SYNBOVICBP	Synthetic cow intestinal calcium-binding protein (ICaBP)
Swiss-Prot.**	A33353	Calcium-binding protein - squid (Watasenia scintillans)
"	S03249	Calcium-binding protein Spec1 - Sea Urchin
"	S40075	Calcium binding protein - tapeworm (Echinococcus granulosus)

* GeneBank® data base with a release date of Oct. 1994. Compiled by the National Institutes of Health and distributed by Hitachi Software Engineering America, Ltd.

** Swiss-Protein data base with a release date of Oct. 1994. Compiled by National Biomedical Research Foundation and distributed by Hitachi Software Engineering America, Ltd.

The calcium binding proteins useful in the processes and plants of the subject invention can also be selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin, calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain, calreticulin, calretinin, calsequestrin, caltractin, gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin. These are listed as class II in Table 3. Further the source of the calcium binding protein can be from bovine, human, mouse or rat calcium binding protein. These are the class I calcium binding proteins listed in Table 3 above.

Phosphate Sinking

It is known in the art that increased phosphate levels further accelerate the activity of the enzymes responsible for photosynthesis. This in turn requires the addition of genes in order to increase the amount of phosphorus or phosphate groups absorbed into and transported to the cytoplasm and chloroplasts of the plant. In addition, increased phosphorus levels may affect the formation rates and/or conversion rates of intermediates required in growing plant tissue. Rao, I.M. et al. (1993) Influence of Phosphorus Limitation on Photosynthesis, Carbon Allocation and Partitioning in Sugar Beet and Soybean, Plant Physiology and Biochemistry, Vol. 31 (No. 2): 223-231.

Some of the proteins that are involved in the sinking and transport of phosphorus include the following proteins from the GeneBank® data base described above: Accession codes Z33142, X76113, B37984, A24265 and M37700.

The hydroxyapatite nucleating protein described herein is also a phosphate binding protein that the present inventors have experimentally verified will cause a greater accumulation of phosphate in the plant.

Metal Binding Proteins

These proteins are termed metal binding proteins because of the their metal sinking activity. Deutsch, H.F. (1984) Primary Structures and Genetic Changes in Mammalian Carbonic Anhydrase Isozymes, By Annals of the NY Academy of Sciences, Vol. 429:183-194.

Various metal and heavy metal binding proteins occur in nature. There are instances of some of these occurring in plants. In plants, these metal binding proteins seem to be adaptations that allow the plant to bind large amounts of toxic metals found in the soil, most notably metals like copper and nickel. Chang, T.K. et al. (1991) Gene Synthesis, Expression, and Mutagenesis of the Blue Copper Proteins Azurin and Plastocyanin, Proc. Natl. Acad. Sci. 88: 1325-1329.

In plants, some metal binding proteins, such as the copper and nickel accumulating proteins, have evolved to sink primarily one metal specifically from the environment. In contrast, heavy metal binding proteins from animal origin and some yeasts (e.g., metallothionein) sink a wide range of monovalent and divalent cations.

In animals, the metal binding proteins include ubiquitin (which is a protein similar to carbonic anhydrases and has a similar ancestral origin) and metallothionein. Both ubiquitin and metallothionein have a wide range of action for sinking monovalent and divalent cations, such as cadmium, copper, zinc and

iron, and heavy metals. Although the amino acid sequence controls the specificity of the metal binding properties, ubiquitin and metallothionein are known to bind a diversity of metals simultaneously. Therefore, both ubiquitin and metallothionein should have particular importance in detoxifying a range of metals in plants. Practical commercial applications resulting from metal binding proteins can have a multiplicity of positive effects, including aiding photosynthesis, increasing crop yields, providing critical minerals for maximizing enzymes and detoxifying toxic heavy metals in plant cells that are absorbed from the environment.

Moreover, in some cases the increased plant growth rates may occur by the metal binding protein aiding in the absorption and transport of other metals and micronutrients. This can be accomplished, for example, by utilizing a protein that binds elements such as zinc and various complexes, such as nitrate and phosphate, in plant tissue. Metallothionein, for example, assists in the transport and absorption of molybdenum, cobalt, selenium, zinc and other metals and metal complexes that are present in low concentrations in soils and plants, and often required for rapid or sustained growth. These particular metals can also be supplied by the use of commercial fertilizers. However, the accumulation of the metals is affected by the pH of the soil or growth medium and the metal binding ability of the plant. Although the metals can be supplied in fertilizers, the proteins need to be present in the plant in order to increase uptake and/or absorption of those metals. If more uptake is needed, the best way to obtain it is to add more sinking or binding capacity in the plants.

The present inventors have discovered that the metal binding protein, metallothionein, enhances the growth

rates of a number of plants. This therefore indicates that the class of metal binding proteins naturally occurring in various animals offers an advantage in growth rates by reducing the level of toxic cations in growing plant cells. The metallothionein proteins, i.e., the alpha and beta subunits, and synthetic genes can be identified by homology to some of the metal binding protein examples disclosed in Table 5. These are intended to be illustrative of the types of metal binding proteins that can be used with the subject invention. They are not, however, intended to limit the invention in any manner.

Table 5

Metallothionein-Like Metal Binding Protein

Data Bank	Accession Code	Brief Description
GeneBank®*	*ELMETL	E. lucius DNA sequence of metallothionein locus
"	HSMNKMBP	H. sapiens MNK MRNA for heavy metal binding protein
"	MOTHIO	Monkey complementary DNA coding for metallothionein
"	NBMETL	N. barbatulus DNA of metallothionein locus
Swiss-Prot.**	MTC_YEAST	Metallothionein (Cu-MT) Precursor
"	CELMTCE1	C. elegans metallothionein-like protein (MT-Ce) MRNA clone
"	SCMAC1	S. cerevisiae gene for MAC1 metal binding activator

* GeneBank® data base with a release date of Oct. 1994. Compiled by the National Institutes of Health and distributed by Hitachi Software Engineering America, Ltd.

** Swiss-Protein data base with a release date of Oct. 1994. Compiled by National Biomedical Research Foundation and distributed by Hitachi Software Engineering America, Ltd.

Other metal binding proteins that are expected to have some beneficial effect on plant growth include ubiquitin, zinc binding proteins, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5, HIV-1 enhancer-binding protein MRNA and *S. cerevisiae* gene of metal binding activator. The inventors expect that any metal binding protein would exhibit this behavior as they would reduce intracellular toxicity to cations.

The short segment of the amino acid sequence of the metallothionein protein that was incorporated into plants in the examples below is identified as SEQ ID NO: 7. It is also disclosed in Pan, A., et al. (1994) Alpha Domain of Human Metallothionein IA Can Bind to Metals in Transgenic Tobacco Plants, Molecular & General Genetics, Vol. 242 (No. 6): 666-674. SEQ ID NO: 8 identifies a nucleotide sequence for the heterologous gene encoding for the metal binding protein, metallothionein, identified as SEQ ID NO: 7.

Biomineralization Proteins

One of the biomineralization proteins of the present invention is hydroxyapatite nucleating protein. Hydroxyapatite [$Ca_5(PO_4)_3$ (F, Cl, OH)] consists mainly of oxygen, phosphorus and calcium and is found in the bones of most animals and is sometimes expressed in bacteria including species of *Cornyobacterium*. It is a solid structural material that is not normally found in plants.

The present inventors have shown that the expression of a hydroxyapatite nucleating protein in tobacco plants increases their growth rates. In other plants, such as trees, it is believed that the expression of the

hydroxyapatite nucleating protein may cause the woody tissue to become stronger or stiffer through the deposition of calcium complexes, thereby reducing bleach requirements during paper pulping. Even without the stiffening effects, the expression of hydroxyapatite nucleating protein in plants produces an increase in cellular calcium and phosphorus absorption and transport which in turn increases their growth rates. Increases in growth rates of plants expressing hydroxyapatite nucleating protein particularly occurs in the presence of a growth medium or soil containing extra or enhanced levels of calcium (such as in the form of lime) and phosphorus (such as in the form of alkali phosphates). Bacteria containing plasmids expressing the hydroxyapatite nucleating protein gene sequences grow rapidly in the presence of media containing enhanced levels of calcium chloride or calcium nitrate and potassium dihydrogen phosphate or similar phosphorus bearing compounds. It is possible that overproduction of hydroxyapatite nucleating protein might be inhibitory to the plant. Therefore, proper promotion of the inserted hydroxyapatite nucleating protein gene is important.

The hydroxyapatite nucleating protein gene developed by the present inventors and used in the examples was based upon the work of Ennever and others. Ennever, J., Vogel, J.J. and Levy, B.M. (1974) Lipid and Bone Matrix Calcification *in Vitro*, Proc. Soc. Expt. Biol. Med. 145: 1386-1388; Ennever, J., Vogel, J.J., Rider, L.J. and Boyan-Salyers, B. (1976) Nucleation of Microbiologic Calcification by Proteolipid, Proc. Soc. Expt. Biol. & Med. 152: 147-150; Ennever, J., Riggan, J., Vogel, J.J. and Boyan-Salyers, B. (1978) Characterization of *Bacterionema matruchotii* Calcification Nucleator, J. Dent. Res. 57(4): 637-642. Ennever et al. discovered that a proteolipid from

Coryneobacter matruchotti nucleated the formation of hydroxyapatite. As a result, Ennever et al. studied this proteolipid and attempted to characterize it, but did not sequence or analyze the protein or determine how it works. Ennever et al. was apparently only interested in studying the metabolic activity of the protein. The present inventors purified this hydroxyapatite nucleating protein and discovered that it consisted of approximately five different proteins that were separable by both HLPC and SDS gradient gel electrophoresis. The present inventors also cloned this protein and used it synthetically. After purifying and hydrolyzing this protein, the present inventors located its nucleating activity on a 23 amino acid sequence that maintained its activity even after side groups, such as the lipid attached to the methionine amino acid residue, were cleaved. This 23 amino acid peptide was then analyzed by amino acid composition, sequence and mass spectra techniques (all three of which enable determination of the sequence of the peptide) and the amino acid sequence was the same using all three techniques. The amino acid sequence of this hydroxyapatite nucleating protein is identified as SEQ ID NO: 1. This peptide has a mass of 2574. Other variants of this peptide seem to share the same active site region, but have a few differences in sequence, most of which are at the carboxy end of the peptide. Prior to this work, it was unknown that there were variants in these five proteins. There are other gene sequences not identical to the hydroxyapatite nucleating protein gene that carry out the same function but the active sites are identical.

The specific methods used for production, purification and analysis of this 23 amino acid hydroxyapatite nucleating protein are as follows:

Fermentation

Corneyobacter matruchotti (ATCC 14266) was grown for 7 days at 37°C in the following fermentation medium in a 40 liter fermentation tank at a pH of 7.4 under aerating conditions.

Fermentation Medium

Ingredient	Concentration
glucose	2.00 g/l
casein hydrolysate	5.00 g/l
sodium carbonate	1.85 g/l
disodium carbonate	0.55 g/l
sodium acetate	0.147 g/l
calcium chloride dihydrate	14.50 mg/l
magnesium sulfate heptahydrate	35.00 mg/l
ferrous sulfate heptahydrate	4.00 mg/l
manganese sulfate	0.15 mg/l
sodium molybdate dihydrate	0.15 mg/l
p-aminobenzoic acid	2.00 mg/l
riboflavin	2.00 mg/l
d-pantothenic acid	2.00 mg/l
myo-inositol	2.00 mg/l
thiamine	2.00 mg/l
nicotinic acid	1.00 mg/l
pyridoxine HCl	1.00 mg/l
biotin	0.10 mg/l
folic acid	0.10 mg/l

Ingredient	Concentration
pimelic acid	0.10 mg/l
thioctic acid	0.10 mg/l
adenine	20.0 mg/l
guanine	20.0 mg/l
thymine	20.0 mg/l
xanthine	20.0 mg/l
tris base	18.2 g/l

Crude purification of the hydroxyapatite nucleating protein was accomplished in the following manner: (1) harvest cells from fermentation tank with tangential flow filter (0.22 μm) and discard supernatant; (2) wash cells with MQ water (18 megaohm, organic and particulate free water) by centrifugation at 10,000 g in a continuous sharples centrifuge for 5 minutes; (3) lyophilize cells; (4) extract dry cells (5 grams in 300 ml of a 2:1 mixture of chloroform:methanol); (5) filter through 0.22 μm filter; (6) wash solvent extract two times with 100 ml of physiological saline; (7) evaporate to 1/8th of volume; (8) extract with 50 ml of acetone; (9) dry and redissolve in 10 ml of a 2:1 mixture of chloroform:methanol; (10) collect peaks (fractions) by LH-20 Sephadex fractionation; and (11) dry fraction and evaluate for hydroxyapatite nucleating protein activity.

The assay for hydroxyapatite nucleating activity contains the following ingredients: 0.276 gm of sodium phosphate, 4.09 gm of sodium chloride, 1.85 gm of sodium bicarbonate, 0.37 gm of potassium chloride and a

quantity of MQ water (18 megaohm, organic and particulate free water) sufficient to make 800 ml.

The pH of the assay solution is adjusted to a pH of 6.6. A 100 ml aliquot of a calcium chloride solution (0.296 g/liter) is added and double distilled water is also added to bring the total volume to 1 liter. Thymol (0.2 g) is added to the solution as a preservative and the entire solution is filter sterilized through a 0.2 micron filter.

To test for hydroxyapatite nucleating activity, one mg of the test material and up to 10 ml of the above-described assay solution are mixed. At 24 to 72 hours, if nucleating activity is present, there should be a deposit in the tube after centrifugation. The precipitate can be washed with 100% ethanol, air dried and analyzed using transmission electronmicroscopy or x-ray diffraction to confirm that the deposit is hydroxyapatite produced only in the presence of the hydroxyapatite nucleating protein. Only the purified protein gave positive results. Negative protein and non-protein controls did not form hydroxyapatite.

Upon electrophoresis of the crude purification extract, a series of bands were discovered. Therefore, primary purification by HPLC was performed showing five bands with hydroxyapatite nucleating protein activity. While there was some differences between the bands as judged by their retention times, one band was selected for sequencing, amino acid and mass times, spectroscopy analysis.

The present inventors also verified the structure by synthesizing the peptide and testing it for nucleation of hydroxyapatite. Antibody tests, bioassay and x-ray data confirmed hydroxyapatite nucleation. Using common

molecular genetic techniques, a synthetic gene having the nucleotide gene sequence identified as SEQ ID NO: 2 was made and inserted into a pUC18 plasmid and electroporated into *Escherichia coli* XL1 MRF'. The nucleating portion of this sequence was found to begin at the eighth codon. This bacterial protein also gave high activity in microscopic assays. The differences in calcium levels between the control and the experimental cells were easily measured using atomic absorption spectroscopy. This test helped confirm that the hydroxyapatite nucleating protein was effective in vivo as well as in vitro. As described in more detail below, for use with plants, the nucleotide gene sequence identified as SEQ ID NO: 2 was inserted into the pBI-121 plasmid for insertion into *Agrobacterium tumafaciens* and was found to transform the plants in all cases. All preparations had a positive antibody reaction with mouse and rabbit antibody made using Freund's adjuvant on the extract before HPLC purification. The x-ray crystallography data of the resulting hydroxyapatite crystallization from the purified protein product was exactly the same as a known standard of hydroxyapatite from dentin.

The insertion of the hydroxyapatite nucleating protein was also found to accelerate or enhance hydroxyapatite deposition and growth rate in plants in the presence of an inserted carbonic anhydrase gene. When those genes were inserted as a pair, the hydroxyapatite nucleating protein and carbonic anhydrase genes elevated the levels of calcium and phosphorus and complexes in plant tissue. Depending on the media and soil composition and the crop or plant in which the expression is occurring, the ratio of calcium to phosphorus in the tissue can vary widely. The theoretical ratio for calcium to phosphorus in pure crystalline hydroxyapatite is 5 to 3, based on the general formula

$Ca_5(PO_4)_3(F, Cl, OH)$. In pure form, the apatites have a hardness of about 5 on the Mohs scale and a specific gravity of about 3.1, which makes them important for potential applications where deposition increases the strength of the plant. Within the scope of this invention, the calcium to phosphorus elemental ratios in green tissue can vary significantly depending on the crop. One of the objects of this invention is to develop plants that have elevated calcium and phosphorus levels that could be used to supplement the diets of individuals who cannot consume dairy products. Animal feeds having enhanced levels of calcium for farm animals can also be obtained by using this gene.

Characterization of the 23 Amino Acid Hydroxyapatite Nucleating Protein

The discovery of a hydroxyapatite nucleating protein which is only 23 amino acid residues in length represents a major advance towards understanding how animals deposit hydroxyapatite. Because most biological systems are so complex, the mechanism of this reaction has not been elucidated to date. However, with hydroxyapatite nucleating protein, the binding sites are predicted to bind multiple calcium and phosphate ions and set up at specific molecular distances for nucleation to occur.

As previously mentioned, the structure of the 23 amino acid hydroxyapatite nucleating protein is identified as SEQ ID NO: 1. This sequence was determined by sequencing the blotted purified protein. A mass of 2574 mass units was found. In addition, mass spectroscopy data determined by MALDI mass spectroscopy of blotted purified protein indicated major peaks at 2592.0 and 2688.9 mass units. Although the first major peak is not the same as the mass (2574), the two differ by a mass of 18. This is explained by an oxidized

methionine group that developed from cleaving the lipid attached at this location in the natural system resulting in an oxidized native protein band. Ennever et al. showed that the native peptide had a fatty acid group, but did not indicate where it was on the protein. Finally, the amino acid composition of the hydroxyapatite nucleating protein was determined from a western blot band. A general method of conforming the identity of a peptide is to check whether sequence, amino acid composition and mass spectroscopy data show good agreement. A good fit with the other two methods occurred in this case.

The peptide was prepared synthetically and purified using high pressure liquid chromatography ("HPLC"). Because the peptide was not soluble in water in the millimolar concentrations needed for nuclear magnetic resonance ("NMR") studies, the present inventors dissolved the protein with organic solvents. The peptide is soluble in 1:1 chloroform:methanol mixtures up to millimolar concentrations. It is also soluble in pure ethanol to micromolar concentrations. Because the three dimensional structure of peptides can depend on the choice of solvent, the degree, if any, to which solvent affects this structure is still being investigated. Based on the work to date, the present inventors believe that the secondary structure of the hydroxyapatite nucleating protein is unchanged in most solvents as enzymatic activity is high even when solvents are present.

Using the synthetic purified peptide made with the structure from sequence data, two-dimensional homonuclear totalized correlated spectroscopy ("TOCSY"), nuclear overhauser enhancement spectroscopy ("NOESY") and double quantum filtered phase sensitive correlated spectroscopy ("DQF-COSY") experiments in

protonated and deuterated solvents (CD_3Cl/CD_3OD and CD_3Cl/CD_3OH mixtures) were conducted. The peptide was dissolved in CD_3Cl/CD_3OD just prior to acquisition of the NMR spectra using a Unity Plus 400 Mhz NMR. In this deuterated solvent, immediate exchange of the most solvent accessible amide (NH) protons was observed. However, a significant subset of the amides was found to be resistant to exchange over a period of 60 hours, during which several TOCSY spectra were acquired. As time progressed, more amide peaks were lost from the spectrum due to further exchange with the solvent. Based on the assignments, the slow-exchanging amide protons coincide with those that belong to the most structured part of the peptide, namely amino acid residues 3 to 14. Amino acid residues belonging to the ends of this region exchange at rates intermediate to those of the core of the structured region (the most slowly exchanging) and those in the unstructured (unfolded) carboxy end of the peptide. These observations provided strong evidence of a folded conformation of the protein in the chloroform/methanol mixtures used in these experiments, wherein a significant number of exchangeable amide protons were involved in secondary structure (hydrogen-bonding) and therefore less accessible to exchange.

Formal two dimensional analyses were performed. Sequential assignment correlates the amide bond separating the proton spin systems from adjacent amino acids. This was accomplished using NOESY spectroscopy. The amide proton (NH) of each residue often exhibits NOESY crosspeaks to its own $C^{\alpha}H$ nucleus and to that of the preceding amino acid residue. Thus, one can "walk" from one amino acid to the next, from NH to $C^{\alpha}H$ to NH in both directions. In extended conformations, only a neighboring peak from nucleating overhauser enhancement NMR (hereinafter "NOE") of the amide to the preceding

$C^{\alpha}H$ nucleus is observed. For example, the NOE of the amide proton of phenylalanine at position 3 to the $C^{\alpha}H$ of the preceding glutamine at position 2 is observed, but not that to its own $C^{\alpha}H$, indicating an extended conformation for the first two residues in the sequence. As a general rule, in structured peptide segments, one observes other NOE connectivities, such as NH(i) to NH(i+1), $C^{\alpha}H$(i) to NH(i+3), which are characteristic of the existence of an alpha helix. In the present case, a NOE was observed between the Hb (2.10 ppm) proton of valine at position 12 and a Ha resonating at 3.70 ppm. This has been assigned to the $C^{\alpha}H$ lysine at position 9.

Positioning along the sequence was accomplished by identifying unique amino acids in the sequence. Most useful in this regard were alanine at position 11, valine at position 12, threonine at position 5 and methionine at position 1. The amide-amide region in a 250 msec mixing time NOESY experiment carried out at a temperature of 30°C was found to contain considerable spatial information. The presence of a significant number of NOESY crosspeaks in this region is further evidence of a stable secondary structure in at least part of the sequence. The assignments of proton resonances for the 23 amino acid peptides are shown in Table 6. The units are ppm from trimethylsilane. The spin systems of the glycine (GLY), lysine (LYS) and phenylalanine (PHE) that remain unassigned in the sequence are listed at the end of Table 6.

Table 6: Resonance assignments for the hydroxyapatite - nucleating peptide*

Residue	NH	Hα	Hβ	Hγ	Hδ	Hε
Met 1	7.71	4.21	1.65, 1.33	2.78		
Gln 2	7.72	4.15	1.98, 1.90	2.20		
Phe 3	8.23	4.31	3.03, 2.97			
Ile 4	7.95	3.57	2.09	1.11, 1.07, 0.97	0.86	
Thr 5	7.54	4.01	3.64	1.09		
Asp 6	8.42	4.05	3.07			
Leu 7	7.87	3.52	1.80, 1.68	1.00	0.79, 0.71	
Ile 8	7.63	3.80	1.70	1.16, 0.92, 0.74	0.48	
Lys 9	7.92	3.71	1.60	1.18	1.30	2.80
Lys 10	8.63	3.85	1.96	1.43	1.93	2.41
Ala 11	8.02	3.96	1.48			
Val 12	8.48	3.57	2.10	0.98, 0.86		
Asp 13	8.31	3.99	3.13			
Phe 14	7.71	4.20	2.84, 2.63			
Phe 15	See below					
Lys 16	See below				2.73	
Gly 17	See below					
Leu 18	7.97	3.71	1.83	1.44	0.82	
Phe 19	See below					
Gly 20	See below					
Asn 21	7.92	3.81	2.64, 1.80			
Lys 22	See below					
Gln 23	7.76	4.19	2.06, 1.86	2.16		
GLY	7.96	3.60, 3.60				
GLY	7.86	3.81, 3.70				
LYS	7.95	3.82	2.73			
LYS	7.94	3.70	1.80			2.79
PHE	8.45	4.22	3.99, 2.57			
PHE	7.71	4.29	3.00, 2.60			

* The units are in ppm from trimethylsilane.

The following discussion of the NOESY data is provided in order to help the reader understand the structure of the peptide and how it works. The discussion gives salient interpretations by amino acid group.

Glutamine at 2: The intra-residue NH-Ha NOE is missing for glutamine at 2. However, a strong sequential NOE to phenylalanine at 3 is observed. The intra-residue NH-Ha of phenylalanine at 3 is also weak. This suggests the existence of an extended conformation on the end of the peptide, i.e., the residue has not been folded. An unknown residue has an NH/Ca crosspiece at 7.34/3.93 ppm, evident in the TOCSY spectrum, although not very intense. No corresponding NOESY crosspiece is observed, also suggesting an extended conformation. However, a NOESY crosspiece is observed from this NH (7.34 ppm) to a resonance at 2.16 ppm. This correlates to a TOCSY spectrum NH resonating at 8.65 ppm, which for the Ca resonance is observed at 4.10 ppm. This is glutamine at 2 from sequential assignments to phenylalanine at 3. Confirming this, the 4.10 ppm Ca gives a TOCSY crosspiece to 2.16 ppm and also another resonance at 1.89 ppm, suggesting a pair of beta protons. An expected crosspiece at 2.16/1.89 is observed, but no other TOCSY crosspeaks are observed.

Leucine at 7: A NOE is observed from the Ha of leucine at 7 (3.47 ppm) to the NH of alanine at 11 (8.03 ppm), which is a $d_{\alpha N}$ (I, I+4) type connectivity that is typical of the existence of an alpha helix structure.

Isoleucine at 8: TOCSY crosspeaks from NH (7.65 ppm) to all expected protons are observed: Ha (3.74 ppm), Hb (1.67), H (1.51, 1.11), Heme (0.83) and Heme (0.42). Most interesting is the NH of isoleucine at 8 which gives a NOE to the Ha of one of the glycine (resonating at 7.95/3.57). This glycine has identical alpha proton

resonances (3.57 ppm). The NH of isoleucine at 8 also appears to make a NOE with the Ha of threonine at 5, which is a $d_{\alpha N}$ (I+3) type connectivity that is typical of the existence of an alpha helix structure.

Alanine at 11: There was a unique alanine residue on the basis of the single beta-methyl resonance. NOE's were observed from the alanine-methyl to an amide at 8.54 ppm of valine at 12 and to a CαH nucleus resonating at 3.46 ppm which is part of an isoleucine spin system. A NOE from the amide of alanine (8.04 ppm) to the CαH at 3.46 is also observed. The intra-residue NH-CαH NOE of the alanine residue is strong. The inter-residue NH-CαH from the alanine-NH to the CαH of the isoleucine residue has medium intensity. The two unique resonances (1.75 and 1.69 ppm) are observed for the CβH protons of this isoleucine residue.

Valine at 12: The NH of valine at 12 (resonating at 8.53 ppm) gives a NOE to a NH resonating at 8.04 ppm, which has to belong to alanine at 11. No other strong NOE is observed between these two residues. The sequential NOE between Ha of alanine at 11 and NH of valine at 12 are very weak. The NOE to Ha of lysine at 9 is stronger. Also observed is a NOE between the NH of lysine at 16 (8.69 ppm) and the Ha of valine at 12, which is a $d_{\alpha N}$ (i, I+4) type connectivity that is typical of the existence of an alpha helix structure. The valine at 12 Ca (3.53 ppm) gives a NOE to a NH resonating at 8.33 ppm. This may be aspartic acid at 13. The Hb of valine at 12 gives a NOE to Ha at 3.67 ppm which has TOCSY crosspiece at 2.76 ppm. This is probably lysine at 9.

Phenylalanine at 14: There is an unusual NOE of the Ha of phenylalanine at 15 to the NH of phenylalanine at 14.

Lysine at 16: There is an unusual NOE from the NH of lysine 16 to the NH of aspartic acid at 13.

Phenylalanine at 19: This residue is assigned by the sequential NOE between its own Ha (4.01 ppm) and the NH of glycine at 20.

Glycine at 20: NOE's are observed from the Hb (3.04 ppm), as well as from the Ha of phenylalanine at 19, to an aromatic proton (6.73 ppm).

Asparagine at 21: See lysine at 22.

Lysine at 22: A corresponding intra-residue NH-Ha NOE is also not observed for the 7.96/4.41 ppm crosspiece observed in the TOCSY. There is a weak NOE of the Ha of this residue to the amide of lysine at 22. This lysine is assigned by elimination as it gives no other NOE's. The 7.96/4.41 ppm also correlates with a resonance at 2.61 ppm, which is consistent with this being asparagine at 21.

It is expected that leucine at position 7 and leucine at position 18 have the same NH/Ha chemical shifts. From this data, the NMR indicates that a portion of the peptide is a alpha helix for the most part from at least amino acid residues at positions 7 through 20. We also suspect folding from the structure which would be a peptide that folds into hairpins. This was confirmed by a Ceo and Faschman analysis of the amino acid sequence secondary structure.

We see that both the model and NMR data predict an alpha helix structure from threonine at position 5 to phenylalanine at position 19 and a turn between the two lysines at positions 9 and 10.

Other biomineralization proteins are also known in plants, bacteria and animals, such as the most well known of these naturally hyperaccumulate copper and nickel is some plants. While most are likely involved in detoxification, they have other functions that may cause increased plant growth rates.

Integration of the Photosynthetic Enhancing Genes into Plants

Each of the carbonic anhydrase, calcium binding protein, metallothionein and hydroxyapatite nucleating protein genes discussed above have been individually or in combination inserted into several crops. Methods to express each of the genes individually and in various combinations including all four together have been developed. For example, each of these genes may be inserted into pBI121 and similar modified vectors, such as pB1121, pB1221 and pGU3850, each with their own promoters. Using polymerase chain reaction methods dramatically reduces the time required to insert multiple genes even when different antibiotic selective markers are utilized. This can be accomplished by allowing one plasmid to contain some or all of the genes and having either all of the genes promoted individually or only one promoter region with one or more selective markers.

The above-described gene sequences are modified so that they can be inserted and expressed in various crops. The result is faster growing plants due to the increase in basic metabolic rates caused by increased transport of carbon dioxide and nutrient element absorption and transport. The carbonic anhydrase II gene has shown the greatest increase in plant growth rates in most of the crops tested. Additionally, inserting the calcium binding protein, hydroxyapatite nucleating protein and

metallothionein genes, particularly in combinations, has further improved or enhanced plant growth rates.

Most of the major agricultural crops tested show improvement in their rate of growth when expressing the above-described genes. In addition, *Populus*, a major forest crop, has also been shown to have faster growth rates when expressing these genes. The techniques and methods of the subject invention are also applicable for increasing the growth rates of fruit and nut trees, as well as other crops.

The genes of this invention may be inserted using a variety of methods including an *Agrobacter* insertion mechanism, electroporation or a biolistic gun approach broadly used by plant genetics. Similarly, other transformation vectors and cassettes, other than the one used in the examples described below, could be substituted with success.

The crops in which the above-described genes can be inserted include, but are not limited to, different varieties of bean, cabbage, carrot, corn, cotton, eggplant, guayule, pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato and wheat, as well as fruit and nut trees, forest product trees such as populars and pines and other crops.

Although the methods and sequences described in the examples below were prepared with one cycle of insertion in one plasmid, it is possible to go through a number of insertion cycles with the same or modified genes using different vectors or the same vector with a different antibiotic marker or other selection markers known to geneticists. For example, one vector may have kanamycin resistance and therefore selection is done on

the basis of increasing the concentration of kanamycin until only transformed tissue survives in the tissue culture. The gene sequence can also be inserted into another vector having carbenicillin resistance. By growing the tissue in plants or shooted plants, even if they are kept in sterile enclosures, reintroduction of the genes can be accomplished in successive cycles. This will result in greater gene activity in the plant. Vectors can also be targeted for insertion in different plant tissues.

Similarly markers which are incorporated to identify proprietary cultivars will be used to screen other products in the marketplace and to insure that such materials are not being used without license. Screening for markers used in making transgenic plants are the most prudent first screening technique. If a transengenic plant marker is present, then one would look for proprietary inserts.

In commercial practice, the insertion of the same gene more than once into the same plant is rarely practiced. However, with the present invention, because basic metabolic cycles affect the rate of growth, multiple insertions of the same gene into the same plant can increase the desired effect. Alternatively, as shown in the examples, multiple proteins may be inserted at the same time using an insertion cassette. In addition, the location of the incorporation of the gene sequences affects overall plant growth rates and crop size at maturity. If insertion occurs beyond three or four cycles of transformation, selection and growth is difficult, and is mainly limited by the spectrum of antibiotic or other selection markers available. In the case of metallothionein, however, an additional selection method is possible simply by using toxic metals such as cadmium as the screening material. This

is accomplished by increasing the levels of these toxic metals in the culture media such that the non-transformed tissue will die or stop growing. In the case of hydroxyapatite nucleating protein, additional selection is possible using very high levels of calcium and phosphorus in the initial callus agar media.

The gene sequences for the target proteins of this invention can be made by different methods. These methods include, but are not limited to, complete synthetic sequencing using ligation of smaller pieces and cutting genomic DNA at selected restriction sites to isolate the basic gene. In the case of shorter sequences made by synthetic methods, slight changes in the gene sequence are possible. In some cases, for example as observed with metallothionein, slight changes in the gene sequence may alter the relative absorption rates of metals and metal ions in complex mixtures.

Once the gene sequences are prepared, they are inserted into plasmids, cultured and isolated, and the gene sequences are then inserted into plant vectors. This procedure is appropriate for insertion of one gene at a time. In contrast, gene pairs can be made using polymerase chain reaction primers and cassettes added to the end terminals. This permits the insertion of both genes simultaneously with one promoter or both separately promoted. Introducing three or four genes into one vector is rarely practiced. If practiced, three or four proteins would be produced. It is possible that the sequential placement in the plasmid of the gene sequences is important. By promoting once or in different areas, the effects of promoter and gene placement within a given plasmid construct can greatly alter the protein production rates. Protein expression of genes in sequence will depend on how far away they

are from the promoter that regulates them. Genes further away may be expressed less frequently than those closer to the promoter.

A general method of gene insertion using a modified *Agrobacterium tumafaciens* 4404X (hereinafter identified as *Agrobacterium tumafaciens)* or other strains used for plant infection is as follows. The gene sequence is amplified using a polymerase chain reaction and isolated and purified on gels. The sequence is then inserted into a plant transformation vector such as pBI121 or modified plasmids. The plasmids are purified and then electroporated into competent Agrobacterium tumafaciens by general genetic engineering techniques. Sambrook, E.F., Fritsch, and Maniatis, T. Molecular Cloning (1989) A Laboratory Manual. Second Ed. Cold Spring Harbor Laboratory Press. Culture plates spiked with kanamycin permit selection of colonies expressing the correct gene. These colonies are then kept in the refrigerator or else added to glycerin and frozen at -20°C or -85°C. Single colonies from the culture plates are then cultured in liquid media.

Different crop tissues depending on the crop are then freshly cut into small pieces and added to the *Agrobacterium tumafaciens* liquid culture. After exposure, the pieces are patted dry on sterile paper towels to remove any *Agrobacterium tumafaciens* left on the surface and then added to media plates with antibiotics to kill the remaining *Agrobacterium tumafaciens*. The concentration and combination of antibiotics used is previously determined in order to avoid killing too much of the plant tissue. After five days, the discs are transferred to other plates containing higher levels of the antibiotic kanamycin which acts as a selective marker. The hormone types and concentrations are changed to permit callus growth

and the formation of shoots. For each crop the media composition is different, but the basic methods are the same for the following crops: tobacco, pea, sunflower, peanut, cabbage, carrot, potato and squash.

For single or multiple gene inserts, a common experimental procedure is to start with ten plates of each gene combination with each plate having 25 (5x5) plant pieces. For example, if inserting three genes, the gene combinations are three single genes, three gene pair combinations and one with all three genes for a total of seven gene combinations or 70 plates each having 25 pieces of callus starts for each crop being tested. The expected gene transformation rate ranges from 1 to 50%. Thus, for each ten pieces started, one to five strong and healthy pieces or explant parts that shoot will subsequently be transferred into culture boxes for rooting. This procedure should result in a sufficient number of healthy transformed plants of each gene type for greenhouse or field trials and provide enough statistical data to determine the effect of the genes on these crops.

Once callus, which is defined as undifferentiated plant tissue, is formed, they are transferred again to plates with different media compositions to control the number and growth rate of the shoots. Within two months from the beginning of transformation, shoots should be about one inch tall, and within three months, several inches tall depending on the crop. Plants are then transferred into larger boxes in order to form roots so that the process of soil planting can begin. The plants are slowly acclimated to normal relative humidity and direct exposure to outside nonsterile air. Prior to this, cultivation occurred in a sterile environment. The correct lighting is very important during this process.

To test for gene expression, one plant is usually sacrificed by cutting a leaf and conducting antibody and polymerase chain reaction testing. For calcium and phosphorus testing, larger sample sizes are needed for atomic absorption measurements of cellular metal concentrations.

Transformation rates are usually determined by the number of pieces or explant parts that survive and form healthy callus and shoots. By using various chemicals including growth hormones, the number of shoots formed from a single callus disk can be controlled. The callus tissue must survive a high level of antibiotics, levels of kanamycin of 100 mg/liter or higher, to insure that all surviving tissue has been transformed. Lower levels of antibiotics lead to mixtures of tissue that can be unstable.

Growth rates can be measured by plant height or weight or leaf area. Depending on the mixture of chemicals and hormones used in the callus and shoot media stages, the crops can be made to have a wide variety of stem heights and leaf area combinations

To make proper comparisons, controls are used with each crop. Control testing should also include crops containing an inserted plant vector without the gene sequence(s). With the present experiments, controls with and without the vector were used. In this way, the transformation process will be the same for both. Differences in growth rates of callus cultures have been observed in as little as two weeks when compared with the above-mentioned control. The best comparison, however, involves growing the plants from seed in controlled blocks in a greenhouse or in field trials. Alternatively, if a faster comparison is necessary, leaf or stem cuttings from the genetically engineered

plants and the control or normal plants can be started at the same time on new culture media without antibiotics or pressure from a selective marker. Although not as quantitative as the comparisons from seed in open field trials, this alternative comparative technique often gives a reliable indication of the expected differences under normal growing conditions.

As previously mentioned, several tests are usually performed on tissue as it progresses from callus to shoot to rooted shoot. These are discussed below and include polymerase chain reaction, antibody and chemical testing.

Polymerase chain reaction testing, for example, involves grinding a small amount of tissue into a powder in liquid nitrogen and then extracting and purifying the genomic DNA. The sample is added to a mixture of polymerase chain reaction reagents and amplified. The reaction products are then subjected to gel electrophoresis. If the inserted gene sequence has incorporated into the plant genome, a band of a specific molecular weight will be produced from the polymerase chain reaction amplification. For each gene transformed, there will be a band of a specific molecular weight which can be observed on the gel.

Antibody testing is used to verify that the gene is present in the DNA and also functioning by producing the desired proteins. This testing takes several days to run but also only requires a small amount of tissue. The antibody is obtained from animals using well established methods. The antibodies developed for calcium binding protein and carbonic anhydrase II bind at high dilutions and have low cross reactivity and high specificity.

Chemical testing includes the determination of the concentration of calcium and phosphorus in samples. However, by using atomic absorption, other metals can also measured to determine their concentrations in the plant tissue and how they might vary as a function of the expressed gene. It was discovered that both calcium binding protein and metallothionein are active proteins and thus the concentration of the basic elements (calcium and phosphorus) in the growth media significantly changes the concentration expressed or absorbed in the plants. Concentration differences of calcium and phosphorus in the growth media appear to make a large differences in expressing these proteins. Thus, very accurate methods must be used to determine the elements present in the growth media and the crops.

Test Gene Sequences

The individual gene nucleotide sequences used in the examples below have been identified as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8. SEQ ID NO: 4 discloses a carbonic anhydrase gene in the human carbonic anhydrase II form. SEQ ID NO: 6 discloses a calcium binding gene that is a synthetic bovine sequence. SEQ ID NO: 8 discloses a synthetic metallothionein gene containing the active region of one example of the natural gene. SEQ ID NO: 2 discloses a synthetic hydroxyapatite nucleating protein gene that was made using knowledge of *Corneyobacter* counterparts. These gene sequences include the special promoters and other components needed to perform all of the above-mentioned tasks, in addition to those needed for polymerase chain reaction testing and insertion into different plasmids. However, the nucleotide sequences mentioned above, i.e., SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8 include only the nucleotide sequence for producing the protein. The

promoters, insertion markers and other elements, which are described in Tables 7-20 below, are all known to those in this art. As previously mentioned, many other specific protein sequences can fulfill the same functions within the confines of providing the same or similar active sites.

In order to insert these basic sequences as pairs or quadruplets, additional sequences called cassettes are required. Cassettes, which are sequences produced entirely by synthetic gene synthesizer equipment, are designed to permit the addition of various restriction sites in order to permit cutting and ligation in different combinations. Tables 7-20 disclose the cassettes that the inventors designed and used for pairing two or more gene sequences in the same vector.

Table 7 discloses the sequence for the combination of all four genes -- calcium binding protein, carbonic anhydrase II, hydroxyapatite nucleating protein and metallothionein -- each of which is separately promoted.

Table 8 discloses the vector construct for the gene pairing of metallothionein and hydroxyapatite nucleating protein.

Table 9 discloses the vector construct for the gene pairing of carbonic anhydrase II and calcium binding protein.

Table 10 discloses the vector construct for the gene pairing calcium binding and hydroxyapatite nucleating protein.

Table 11 discloses the vector construct for the gene pairing of carbonic anhydrase II and metallothionein proteins.

Table 12 discloses the vector construct for the gene pairing of calcium binding protein and metallothionein proteins.

Table 13 discloses the vector construct of the gene pairing of carbonic anhydrase II and hydroxyapatite nucleating proteins.

Table 14 discloses the vector construct for the gene pairing of calcium binding, metallothionein and hydroxyapatite nucleating proteins.

Table 15 discloses the vector construct for the gene pairing of calcium binding, carbonic anhydrase II and metallothionein proteins.

Table 16 discloses the vector construct for the gene pairing of calcium binding, carbonic anhydrase II and hydroxyapatite nucleating proteins.

Table 17 discloses the vector construct for inserting the hydroxyapatite nucleating protein gene into a pBI121 cassette.

Table 18 discloses the vector construct for inserting a calcium binding protein gene into a pBI121 cassette.

Table 19 discloses the vector construct for inserting the metallothionein gene into a pBI121 cassette.

Table 20 discloses the vector construct for inserting the carbonic anhydrase II gene into a pBI121 cassette.

In any of the above-described cases, the sequence is interchangeable meaning the sequence of two or more genes could be switched in the plasmid ring structure because the proteins are promoted individually. The order of these sequences can affect their relative expression levels. An alternative approach is to use only one promoter in the plasmid. In this approach, the placement of the gene sequences is more important because one protein may be preferentially produced over another further from the promoter site.

While Table 7 discloses all four genes inserted at once with only one promoter, the vector construct can be altered by removing the promoters or adding others, such as adding promoters between each of the genes.

The present inventors experimented with many permutations of the above-described and other gene cassettes. The specific examples are described below for tobacco, potato tomato and *Populus* plants.

Examples of Gene Insertion

The process of inserting the gene sequences of this invention into crops can be carried out in the following manner. Tobacco, cultivar NC-72, had the following five gene sequences or combinations of gene sequences inserted into a pBI121 plant transformation cassettes:

1. Carbonic anhydrase II from human red blood cells (SEQ ID NO: 4);

2. Calcium binding protein from bovine intestine synthetic sequence (SEQ ID NO: 6);

3. Hydroxyapatite nucleating peptide synthetic sequence relating to *Corneyobacter* (SEQ ID NO: 2);

4. Metallothionein peptide synthetic gene (active region only) (SEQ ID NO: 8); and

5. The combination of carbonic anhydrase II and calcium binding protein (SEQ ID NO: 4 and SEQ ID NO: 6).

These same sequences have been tried in other plant systems, as outlined below.

Example 1 (Tobacco)

Development of Insertion Cassettes

The following part of Example 1 was a common step to all the examples. Therefore, this description is not repeated for the other crop examples.

The carbonic anhydrase II gene sequence was isolated from human carbonic anhydrase. From the gene sequence, the polymerase chain reaction primers shown below as SEQ ID NO: 9 and SEQ ID NO: 10 were synthesized in order to amplify the gene and add new restriction sites, specifically Xba I at the 5' end and Sma I at the 3' end. After polymerase chain reaction amplification, the product was digested with Xba I and Sma I restriction enzymes to obtain overhanging cohesive ends. Digestion with restriction enzymes allowed the ligation of the carbonic anhydrase II gene into the pBI121 plant transformation vector at the Xba I and Sma I sites ensuring that the insert was ligated in the proper orientation.

The carbonic anhydrase II primers discussed in the preceding paragraph are as follows:

Carbonic anhydrase II forward primer (SEQ ID NO: 9) is:
5' GGATCCTCTA GAATGTCCCA TCACTGGGGG TACGGCAAAC AC 3'

Carbonic anhydrase II reverse primer (SEQ ID NO: 10) is:
5' CCCGGGGAGC TCTTATTTGA AGGAAGCTTT GATTTGCCTG 3'

The recombinant pBI121 plasmid containing the carbonic anhydrase II gene insert was electroporated into competent *Agrobacterium tumafaciens* and the cells were plated onto media that contained kanamycin as a selective marker. The individual bacterial colonies that grew on the selective media plates were transferred to liquid media with kanamycin. These liquid cultures were grown overnight at 30°C and were used to isolate large quantities of the recombinant plasmid that had been electroporated into them. Using the above-described primers (SEQ ID NO: 9 and SEQ ID NO: 10) that were specific for the carbonic anhydrase II insert, the isolated plasmid was used as the template in a polymerase chain reaction in order to verify that the gene was actually in the plasmid. If the carbonic anhydrase II gene had been successfully inserted into the pBI121 plasmid, then this polymerase chain reaction would amplify it. The polymerase chain reaction product was then run out on a 0.7% agarose gel visualized with ethidium bromide to verify the presence and size of the product. If the gel band is the same size as the original carbonic anhydrase II gene, verification is deemed positive and insertion of the gene into the plasmid is successful. In order to insure formation of the correct active proteins, other verification techniques including protein analysis by gel electrophoresis and antibody testing were conducted.

This same polymerase chain reaction amplification procedure, insertion into the pBI121 plasmid and polymer chain reaction verification were carried out for the three other genes (calcium binding protein, hydroxyapatite nucleating protein and metallothionein).

Once each of the genes was inserted individually into the plasmid, work could begin on the combinations. In order to link genes together, each of the genes needed unique restriction sites at the beginning of the promoter and the end of the terminator. The restriction site at the end of the terminator of the first gene in the sequence needs to be the same as the restriction site at the beginning of the promoter of the gene that is to follow. These unique restriction sites were added to the carbonic anhydrase II and calcium binding protein sequences by means of polymerase chain reaction cassettes with restriction site overhangs.

The cassettes shown below and identified as SEQ ID NO: 11 through SEQ ID NO: 16 were designed to perform the following functions: (i) contain the required unique restriction sites, (ii) ligate to the genes at specific sites (Hind III at the 5' or promoter end and EcoR I at the 3' or terminator end) after the genes had been digested from pBI121, and (iii) serve as priming sites for polymerase chain reaction amplification and verification. To prepare the genes for cassette ligation, they each had to be digested from the pBI121 plasmid with HindIII and EcoRI restriction enzymes. Once the genes were free from the plasmid, the cassettes could be ligated to the ends of the gene. To verify that the cassettes were attached properly, the ligation mixture was amplified with polymerase chain reaction using primers designed to anneal to a specific sequence on the cassettes. The ligation conditions used are described in Sambrook, E.F., Fritsch, and Maniatis, T. Molecular Cloning, (1989) A Laboratory Manual, Second Ed. Cold Spring Harbor Laboratory Press, which is incorporated by reference. The polymerase chain reaction protocol and reagents were from Panvera in Madison, WI, the Takara Kit RR001A. The optimized

polymerase chain reaction cycling protocol consisted of an initial dwell of 94°C; a cycle of the following steps: (i) 94°C for 30 sec., (ii) 55°C for 2 min. and (iii) 72°C for 3.5 min.; and an ending dwell of 4°C until the reaction mixture could be removed for subsequent steps. To determine if ligation was successful, the polymerase chain reaction product was run on a 0.7% agarose gel using ethidium bromide for visualization of a band. The genes with cassettes were than digested with the appropriate restriction enzymes using restriction protocols of Sambrook, et al. (1989). For carbonic anhydrase those enzymes were Kpn I and EcoRI. For the calcium binding protein, the enzymes were HindIII and Kpn I.

Cassette's for Gene Combinations

Hind III Sal I (SEQ ID NO: 11):

5' GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGGTCGA CAGCT 3'

3' CATGTATTAC AGCAATCTTG CGCATTATGC TGAGTGATAT CCCTCCAGCT GTCGA 5'

Hind III Hpa I (SEQ ID NO: 12):

5' GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGGTTAA CAGCT 3'

3' CATGTATTAC AGCAATCTTG CGCATTATGC TGAGTGATAT CCCTCCAATT GTCGA 5'

Hind III Kpn I (SEQ ID NO: 13):

5' GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGGGTAC CAAGCT 3'

3' CATGTATTAC AGCAATCTTG CGCATTATGC TGAGTGATAT CCCTCCCATG GTTCGA 5'

EcoRI Sal I (SEQ ID NO: 14):

5' GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGAGTCG ACGAATT 3'

3' CATGTATTAC AGCAATCTTG CGCATTATGC TGAGTGATAT CCCTCTCAGC TGCTTAA 5'

EcoRI Hpa I (SEQ ID NO: 15):

5' GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGAGTTA ACGAATT 3'

3' CATGTATTAC AGCAATCTTG CGCATTATGC TGAGTGATAT CCCTCTCAAT TGCTTAA 5'

EcoRI Kpn I (SEQ ID NO: 16):

5' GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGAGGTA CCGAATT 3'

3' CATGTATTAC AGCAATCTTG CGCATTATGC TGAGTGATAT CCCTCTCCAT GGCTTAA 5'

After digestion, the genes were ligated together at the Kpn I site and then inserted back into the pBI121 plasmid at the HindIII (5') and EcoRI (3') sites. The plasmid with carbonic anhydrase II and calcium binding protein linked together as inserts was electroporated into 100 μl of log phase *Agrobacterium tumafaciens* electrocompetent cells and 1-5 μl of 0.1 μg/ml cassette (or plasmid) DNA using a BioRad Gene Pulser® with a 0.2 cm gap cuvette at 1 kvdc and using a 25 uf capacitor and 200 Ω and verified with polymerase chain reaction as described above. Once colonies have been verified for the proper plasmid and insert, the plant infection and transformation can begin.

Infection to transform the plant tissue with the desired inserts was performed by the modified procedure from Horch et. al. (1985) A Simple and General Method for Transferring Genes into Plants Science, Science, Vol. 227: 1229-1231, which is incorporated by reference. Young tobacco leaves were cut from the top of the mature plant and rinsed under running water. The leaves were placed into 70 % ethanol for 5 minutes, a 1:4 solution of sodium hypochlorite and water for 10 minutes and then rinsed three times in sterile water. Using a sterile hole punch, 8 mm discs were cut from the leaves and immersed in an overnight culture of *Agrobacterium tumafaciens* containing the pBI121 plasmid with the carbonic anhydrase II, calcium binding protein, metallothionein and hydroxyapatite nucleating protein inserts for 15 minutes. The discs were dried on sterile paper towels and plated top down onto callus and shoot initiation media, which is described in Tables 21 and 22. After 48 hours the discs were

removed and washed three times in liquid media, patted dry on paper towels as before and plated top down onto callus and shoot media containing 500 mg/l of carbenicillin (see Tables 21 and 22). After another 48 hours the discs were transferred to the same media containing 500 mg/l of carbenicillin and 100 mg/l of kanamycin. The discs were transferred to fresh media every three weeks. Shoots began to appear within two months and were excised from the leaf tissue and placed into Magenta™ boxes containing the shoot elongation media disclosed in Table 22. When the shoots reached 5 - 6 cm in height (which took another 2-3 months), they were transferred to root initiation media described in Table 23 containing 75 mg/l of kanamycin. Once enough root tissue had grown (in about 1-2 months), the plantlets were removed from the tissue culture media and placed into sterile soil and acclimated to normal relative humidity. After 7 to 8 days the plants were transferred to larger pots and placed in the greenhouse. These plants were subsequently followed all the way to seeding and were also monitored in subsequent generations for stable insertion of the gene inserts. A stable insert is considered to be three generations with insertion stability.

Results of Example 1 (Tobacco)

The insertion of the carbonic anhydrase into tobacco greatly accelerated the growth rate compared to control plants, which either did not have the carbonic anhydrase enzyme in an inserted plasmid or had no plasmid at all. There was a small but consistent increase in growth over the controls with separate insertion of the calcium binding protein and hydroxyapatite nucleating protein inserts in separate plants. Finally, a positive synergistic increase in growth was observed over the controls with the double

inserts, i.e., with the carbonic anhydrase II and calcium binding protein genes linked together in the same plasmid.

Compared to the controls, separate insertion of the calcium binding protein and hydroxyapatite nucleating protein increased the calcium levels in the plant leaves by a factor of ten without supplementation of calcium in the growth medium, and up to 50 fold with calcium supplementation in the soil in the form of $Ca(NO_3)_2.H2O$, during growth in pots in a greenhouse environment. There was also a similar stoichiometric increase in the phosphorus levels with the hydroxyapatite insert in the plants.

The addition of the metallothionein insert to tobacco allowed for a variation in the selection procedure whereby 350 μM cadmium (added as cadmium sulfate) could be added to the media for tissue culture described in Tables 21 and 22 and subsequently to the soil. At this level, all the control plants quickly were killed, while no toxicity was observed with the test plants. These results showed up to a 100 fold increase in cadmium uptake over the controls. The insert also seems to similarly protect the plant from many other heavy metals.

Example 2 (Potato)

Potatoes were infected by modifying, as described below, the procedure of Snyder and Belknap (1993), A Modified Method for routine Agrobacterium-mediated Transformation of in vitro grown potato microtubers. Plant Cell Reports 12: 324-327, which is incorporated herein by reference. Explant preparation began by surface sterilizing a potato tuber, which was a Russet Burbank cultivar, in 70% ethanol for 5 minutes and then

30 minutes in a sodium hypochlorite solution (1:4 dilution commercial bleach to water). The tissue was rinsed 3 times in sterile water and sterile cork borers were used to create potato cylinders. The potato cylinders were sliced into discs about 1 to 2 mm thick with a scalpel and incubated for 20 minutes in 5 ml of an overnight *Agrobacterium tumafaciens* culture containing the pBI121 plasmid with cassette inserts 1-5 (mentioned above in the beginning of the section entitled "Examples of Gene Insertion") or a control without an inserted gene. The discs were removed from the bacteria and patted dry on sterile paper towels to remove the excess *Agrobacterium tumafaciens*. The discs were then plated onto the callus initiation media disclosed in Table 24 and incubated at 25°C in the dark. After 48 hours the discs were rinsed 3 times in sterile water, patted dry and plated onto callus initiation media disclosed in Table 24 containing 500 mg/l of carbenicillin and returned to the incubator. They remained on that media for 7 days and then were transferred to callus initiation media described in Table 24 with 500 mg/l of carbenicillin and 100 mg/l of kanamycin. When the first signs of callus appeared, in approximately two weeks, the discs were transferred to the shoot initiation media disclosed in Table 25 and placed under growth lights. As the shoots grew they were moved into Magenta™ (Sigma Chem. Co. St. Louis MO) boxes containing the same media. The discs stayed on this media until they had grown large enough to initiate rooting (about 2-3 months) when they were transferred to half strength media with no growth regulators. Once rooting had taken place the agar was carefully removed from the roots and the plantlets were placed into sterile soil and slowly acclimated to normal relative humidity. After 7 to 8 days of gradual acclimation the plants were taken to the greenhouse and grown normally.

Results of Example 2 (Potato)

The insertion of the carbonic anhydrase II into potato accelerated the growth rate of the transformed potatoes over that of the potato controls. There was a small but consistent increase in the growth observed with separate insertion of the calcium binding protein and hydroxyapatite genes. Finally, there was a positive synergistic increase in growth with double inserts of carbonic anhydrase II and calcium binding protein.

The insertion of the calcium binding protein and hydroxyapatite increased the calcium levels up to ten fold without supplementation and up to 50 fold with calcium supplementation in the soil as described above. There was also a similar stoichiometric increase observed for phosphorus levels with the hydroxyapatite nucleating protein insert.

The addition of the metallothionein insert to potato allowed for a variation in the selection procedure whereby 350 μM cadmium added as cadmium sulfate could be added to the media for tissue culture disclosed in Table 9 and subsequently to the soil. At this level, all the control plants were quickly killed, while no toxicity was observed with the test plants. These results showed up to a 100 fold increase in cadmium uptake over the controls, which consisted of one with an inserted plasmid with no gene and one with no plasmid. The insert also seemed to similarly protect the plant from many other heavy metals.

Example 3 (Tomatoes)

The tomato procedure for tissue culture and infection was modified, as discussed below, from the procedure of Katia et. al. (1993) Enhanced Transformation of Tomato Co-cultivate with Agrobacter tumefaciens C58CIRifr::pGSFR1161 in the Presence of Acetosyringone. Plant Cell Reports 12: 422-425, which is incorporated herein by reference. Mature seeds, which were the HyPeel 2196 cultivar, were surface sterilized in 70 % ethanol for 10 minutes, in a bleach solution for 30 minutes and then rinsed three times in sterile distilled water. The seeds were then placed into tubes containing sterilized potato dextrose agar and allowed to germinate and grow for eight days.

The leaves were removed from the sterile seedlings and incubated for ten minutes in a logarithmic culture of *Agrobacterium tumafaciens* containing the pBI121 plasmid and inserts 1-5 (mentioned above in the beginning of the section entitled "Examples of Gene Insertion") in order to infect them. They were then dried with sterile paper towels before being plated onto callus and shoot media. The leaves were co-cultivated for 24 hours with the *Agrobacterium tumafaciens*, then transferred to callus and shoot media disclosed in Table 26 containing 500 mg/l of carbenicillin and 50 mg/l of kanamycin. After three weeks, they were moved to the same media with 500 mg/l of carbenicillin and 75 mg/l of kanamycin. Every three weeks the tissue was transferred to a new plate with the same media until shoots began to form which took approximately an additional 3 to 6 weeks. The shoots were excised from the remaining necrotic leaf tissue and placed in Magenta™ boxes with callus and shoot media described in Table 26 containing 500mg/l of carbenicillin and 100 mg/l of kanamycin. The shoots continued to grow until

they reached 4 - 6 cm in height (in about 1 - 2 months) and then they were transferred to root initiation media disclosed in Table 27 with 100 mg/l of kanamycin. When enough root mass had grown, in approximately 2-3 weeks, the plantlets were transferred to soil and slowly acclimated to a normal relative humidity (approximately 40%) over 7-8 days. After adjusting to normal conditions, the plants were transferred to larger pots and moved to the greenhouse.

The results with the tomato were very consistent to the work with the other two plants above. The transformed plants and the plants from the first generation after seeds seemed to grow faster with carbonic anhydrase II, calcium binding and hydroxyapatite nucleating protein genes separately inserted an inserted in combination.

Example 4 *(Populus)*

The method used with the *Populus* tree was a modification of the procedure of Gerry Tuskan and Connie Wong (personal communication), which is described below. Young shoots were removed from trees growing in the greenhouse. The shoots were stripped of leaves and surface sterilized in 70 % ethanol for 5 minutes, in a bleach solution for 20 minutes and then rinsed three times in sterile water. The shoots were cut into 2-4 mm thick discs to avoid the internode tissue. The discs were incubated for 15 minutes in an overnight culture of *Agrobacterium tumafaciens* containing the pBI121 plasmid with the carbonic anhydrase II and calcium binding protein and then patted dry on sterile paper towels. The discs were co-cultivated for 48 hours on *Populus* callus initiation media disclosed in Table 28, rinsed three times in liquid media, dried as before, and plated onto *the same Populus* callus initiation media containing 500 mg/l of

carbenicillin for an additional 3 days. The disks were then transferred to *Populus* shoot initiation media disclosed in Table 29 containing 500 mg/l of carbenicillin and 75 mg/l of kanamycin. The discs were then transferred to fresh media every three weeks. Shoots began to appear after two months of culture and were moved to Magenta™ boxes with the same media. When the shoots reached 6-10 cm in height, in approximately another 1-2 months, they were transferred to root initiation media disclosed in Table 30. When enough root mass had developed the plantlets were removed from the media and placed into soil where they acclimated to normal relative humidity over ten days. Once acclimated, the plants were transferred to larger pots and moved to the greenhouse.

Results of Example 4 *(Populus)*

The insertion of the carbonic anhydrase II into *Populus* accelerated the growth rate compared to control plants. There was a small but consistent increase in the growth with the separately inserted calcium binding protein and hydroxyapatite nucleating protein genes. Finally, there was a positive synergistic increase in the growth with the double inserts of the calcium binding protein and hydroxyapatite nucleating protein genes.

The insertion of the calcium binding protein and hydroxyapatite increased the calcium levels varying degrees and up to five-fold higher with calcium supplementation in the soil as described above. There was also a similar stoichiometric increase in phosphorus levels with the hydroxyapatite insert.

Growth Conditions

The addition of small amounts of lime (calcium) or N-P-K (nitrogen-phosphorus-potassium) fertilizers may be used with the above-described gene inserts to further maximize the enhancement of growth. It is a common agronomic practice to adjust soil pH and add limiting nutrients. Adding phosphorus, calcium and zinc is of special importance for maximizing the enhancement of photosynthesis of the gene combinations of the present invention. Nitrogen, phosphorus and potassium are considered the major macronutrients in crop growth. The secondary elements are sulfur, calcium and magnesium which also must be present in most major agricultural crops. The micronutrients include boron, cobalt, copper, iron, manganese, molybdenum and zinc. Deficiencies in one or more of these macronutrients, secondary elements or micronutrients can often have a major negative impact on crop growth. This is especially true when photosynthesis enhancing genes are added. For example, in some fruit trees, the combination of manganese and magnesium deficiencies in soil can have a major negative impact on root and tree growth rates. The present inventors have discovered that is particularly helpful to add calcium (either as lime, calcium hydroxide, calcium phosphate or other calcium compounds) to the growth medium when a metal binding protein has been inserted.

Products and Applications

Using the inventions as described herein there are a number of plant products that can be engineered for large scale applications. Several examples of these products are described as follows:

Nutriceuticals

There are a number of products marketed which are in the form of dried crops such as beets, carrots, flowers and other plants. These are in the form of free flowing powders, tablets and capsules. These products are sold for various medically related reasons such as vitamin or mineral content. Using the gene sequences disclosed herein, a number of crops can be made to express high levels of calcium and phosphorus. These would be beneficial to people who have a low tolerance for dairy products. These are anticipated to be more bioavailable than mineral supplements alone. To obtain high mineralization vegetables, the genes to insert are the calcium binding protein and hydroxyapatite. In fact, most known major crops could be made to express these genes and produce crops with elevated levels of minerals and elements which could provide a distinct market competitive advantage.

In addition, there are a number of nutriceutical products which are sold for high levels of different forms of chromium, selenium and other elements and compounds. A number of these metals cannot be applied to the soil because it is against various environmental protection agencies regulations to add or supplement the levels of these metals in the soil. However, crops such as carrots, beets, and other tubers can be grown hydroponically with elevated levels of these metals in the nutrient solutions which will accumulate in the plants. These tubers can then be dried and sold in the nutriceutical market as materials which have metals in high levels and are "bioavailable". This unique property of the plants produced using this invention relates to elements which are released into the system and absorbed more readily than materials in which the element is in pure powdered form. The genes to insert

for these type of products are the metallothionein which may also be coupled with the calcium binding protein in the same plasmid.

Trees

Using the process of the present invention will also lead to a number of improved tree lines including hard and softwoods. Trees could be made to grow faster using the carbonic anhydrase II gene. Trees, which are lower in value by virtue of producing cellulosics that are "soft" or that have woods that are low in tensile and compressive strengths, are anticipated to be engineered to have stronger wood via insertion of the hydroxyapatite nucleating protein gene. By combining several of the genes of this invention, woods can be made which should have higher strength, in trees growing at a faster rate. To optimize the effects of this product, lime can be added to the soils either directly or through irrigation systems. Trees expressing the gene calcium binding protein and/or hydroxyapatite nucleating protein also should have the added benefit that they will burn cleaner due to the higher levels of calcium in the wood. Further, for manufacturing processes involving pulp, the requirement for bleach should also be lower and the trees should grow in nutrient deficient soils.

There are a number of tree varieties including pines which are severely affected by some metals in the soil including aluminum and others. Those pines which express the metallothionein gene however, either alone or in combination with the calcium binding protein and/or hydroxyapatite gene, should have a built in resistance to this type of metal poisoning and thus the trees may appear to grow faster in a given area. The affect however would be more based on reducing the

effects of acid rain metal poisoning than increased growth rates caused by the carbonic anhydrase II gene or the carbonic anhydrase II gene in combination with the calcium binding protein gene.

Crops

Using the genes of this invention specifically the carbonic anhydrase II gene alone which may be in combination with the other genes, it is expected that most major crops will grow at a faster rate. In some cases the product advantage relates to reduced time to reach maturity such as tomatoes in which processing or manufacturing facilities are idle for a large portion of the year until there are enough tomatoes ready for processing. In other cases, larger crop yields will be produced if they are given the same length of time to grow as crops without the gene inserts.

There are a number of ways in which seeds can be produced for the major crops. These can be hybrids or pure lines in which the desired trait will be passed down to future generations. Hybrid seeds are often preferred as a means of protecting the proprietary interests of the company owning the rights to the new inserts. Given the potential large variation in how the seed lines are developed and the number of gene combinations of this invention, the level of expression and the type of impact on the particular crop may vary widely. In some cases the effects are also tied to whether or not nutrients and fertilizers are added to the crop during its growth cycle, wherein concentration and timing may also vary widely.

Table 7. Combination of all four genes (carbonic anhydrase II, calcium binding protein, hydroxyapatite nucleating protein and metallothionein).

Base Nos.	Description
1-817	35S Promoter
818-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2232	Restriction Sites
2233-2337	Metallothionein Gene
2338-2590	NOS Terminator
2591-2608	Restriction Sites
2609-3420	35S Promoter
3421-3438	Restriction Sites
3439-3525	Hydroxyapatite Nucleating Protein Gene
3526-3778	NOS Terminator
3779-3796	Restriction Sites
3797-4608	35S Promoter
4609-4626	Restriction Sites
4627-5421	Carbonic Anhydrase II Gene
5422-5680	NOS Terminator

Table 8. Gene pairing for hydroxyapatite nucleating and metallothionein proteins.

Base Nos.	Description
1-818	35S Promoter
819-837	Restriction Sites
838-924	Hydroxyapatite Nucleating Protein Gene
925-1177	NOS Terminator
1178-1195	Restriction Sites
1196-2007	35S Promoter
2008-2019	Restriction Sites
2020-2124	Metallothionein Gene
2125-2383	NOS Terminator

Table 9. Gene pairing for calcium binding and carbonic anhydrase II proteins.

Base Nos.	Description
1-818	35S Promoter
819-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2238	Restriction Sites
2239-3033	Carbonic Anhydrase II Gene
3034-3292	NOS Terminator

Table 10. Gene pairing for calcium binding and hydroxyapatite nucleating proteins.

Base Nos.	Description
1-818	35S Promoter
819-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2238	Restriction Sites
2239-2325	Hydroxyapatite Nucleating Protein Gene
2326-2584	NOS Terminator

Table 11. Gene pairing for carbonic anhydrase II and metallothionein proteins.

Base Nos.	Description
1-818	35S Promoter
819-837	Restriction Sites
838-1632	Carbonic Anhydrase II Gene
1633-1885	NOS Terminator
1886-1903	Restriction Sites
1904-2715	35S Promoter
2716-2727	Restriction Sites
2728-2832	Metallothionein Gene
2833-3091	NOS Terminator

Table 12. Gene pairing for calcium binding and metallothionein proteins.

Base Nos.	Description
1-818	35S Promoter
819-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2332	Restriction Sites
2333-2337	Metallothionein Gene
2338-2596	NOS Terminator

Table 13. Gene pairing for carbonic anhydrase II and hydroxyapatite nucleating proteins.

Base Nos.	Description
1-818	35S Promoter
819-837	Restriction Sites
838-1632	Carbonic Anhydrase II Gene
1633-1885	NOS Terminator
1886-1903	Restriction Sites
1904-2715	35S Promoter
2716-2733	Restriction Sites
2734-2820	Hydroxyapatite Nucleating Protein Gene
2821-3079	NOS Terminator

Table 14. Gene pairing for calcium binding, metallothionein and hydroxyapatite nucleating proteins.

Base Nos.	Description
1-818	35S Promoter
819-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2232	Restriction Sites
2233-2337	Metallothionein Gene
2338-2590	NOS Terminator
2591-2608	Restriction Sites
2609-3420	35S Promoter
3421-3438	Restriction Sites
3439-3525	Hydroxyapatite Nucleating Protein Gene
3526-3784	NOS Terminator

Table 15. Gene pairing for calcium binding, carbonic anhydrase II and metallothionein proteins.

Base Nos.	Description
1-818	35S Promoter
819-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2238	Restriction Sites
2239-3033	Carbonic Anhydrase II Gene
3034-3286	NOS Terminator
3287-3304	Restriction Sites
3305-4116	35S Promoter
4117-4128	Restriction Sites
4128-4233	Metallothionein Gene
4234-4492	NOS Terminator

Table 16. Gene pairing for calcium binding, carbonic anhydrase II and hydroxyapatite nucleating proteins.

Base Nos.	Description
1-818	35S Promoter
819-836	Restriction Sites
837-1137	Calcium Binding Protein Gene
1138-1390	NOS Terminator
1391-1408	Restriction Sites
1409-2220	35S Promoter
2221-2238	Restriction Sites
2239-3033	Carbonic Anhydrase II Gene
3034-3286	NOS Terminator
3287-3304	Restriction Sites
3305-4116	35S Promoter
4117-4134	Restriction Sites
4135-4221	Hydroxyapatite Nucleating Protein Gene
4222-4480	NOS Terminator

Table 17. Hydroxyapatite nucleating protein gene inserted into a pBI121 cassette.

Base Nos.	Description
1-181	pBI121
182-999	35S Promoter
1000-1017	Restriction Sites
1018-1104	Hydroxyapatite Nucleating Protein Gene
1105-1357	NOS Terminator

Table 18. Calcium binding protein gene inserted into a pBI121 cassette.

Base Nos.	Description
1-181	pBI121
182-999	35S Promoter
1000-1017	Restriction Sites
1018-1318	Calcium Binding Protein Gene
1319-1571	NOS Terminator

Table 19. Metallothionein protein gene inserted into a pBI121 cassette.

Base Nos.	Description
1-181	pBI121
182-999	35S Promoter
1000-1017	Restriction Sites
1018-1116	Metallothionein Gene
1117-1369	NOS Terminator

Table 20. Carbonic anhydrase II gene inserted into pBI121 cassette.

Base Nos.	Description
1-181	pBI121
182-999	35S Promoter
1000-1017	Restriction Sites
1018-1812	Carbonic Anhydrase II Gene
1813-2162	NOS Terminator

Table 21

Tobacco Callus or Callus Initiation Media

Murashige & Skoog salts[1/]	1 liter packet
sucrose	30 gm
B-5 vitamin stock100X[2/]	10 ml
naphthalene acetic acid	0.1 mg
benzyl amino purine	1.0 mg
agar	8 gm
pH	5.7
distilled water to	1 liter

[1/] Sigma Cat. #M-5524

[2/] Gamborg B-5 vitamin mixture (Sigma G1019) (100x means concentrated 100 fold)

Table 22

Tobacco Callus Initiation Shoot Medium

Murashige & Skoog salts[1/]		1 liter packet
sucrose		30 gm
B-5 vitamin stock 100X[2/]		10 ml
kinetin		1 mg
agar		8 gm
pH		5.7
distilled water	to	1 liter

[1/] Sigma Cat. #M-5524

[2/] Gamborg B-5 vitamin mixture (Sigma G1019) (100x means concentrated 100 fold)

Table 23

Tobacco Callus Initiation Root Medium

Murashige & Skoog salts[1/]		1 liter packet
Sucrose		30 gm
B-5 vitamin stock 100x[2/]		10 ml
gar		8 gm
pH		5.7
distilled water	to	2 liters

[1/] Sigma Cat. #M-5524

[2/] Gamborg B-5 vitamin mixture (Sigma G1019) (100x means concentrated 100 fold)

Table 24

Potato Callus Initiation

Murashige & Skoog salts[1/]	1 liter pkt
sucrose	30 gm
naphthalene acetic acid	0.19 mg
benzyl amino purine	2.3 mg
giberillic acid	1.0 mg
agar	7.0 gm
myo-inositol	50 mg
nicotinic acid	0.25 mg
pyridoxine HCl	0.25 mg
thiamine HCl	0.25 mg
glycine	1.0 mg
casein hydrolysate	0.5 mg
distilled water to	1000 ml
pH	5.8

Sigma Cat. #M-5524

Table 25

Potato Shoot Initiation

	Murashige & Skoog salts[1/]	1 liter pkt
	sucrose	20 gm
	zeatin	2.19 mg
	agar	8.0 gm
	myo-inositol	50 mg
	nicotinic acid	0.25 mg
	pyridoxine HCl	0.25 mg
	thiamine HCl	0.25 mg
	glycine	1.0 mg
	casein hydrolysate	0.5 mg
	distilled water to	1000 ml
	pH	5.8

[1/] Sigma Cat. #M-5524

Table 26

Tomato Callus & Shoot Medium

Murashige & Skoog salts[1/]		1 liter pkt
Murashige & Skoog vitamins[2/]		1 ml
sucrose		30 gm
benzyl amino purine		2 mg
agar		8 gm
pH		5.8
distilled water	to	1 liter

1/ Sigma Cat. #M-5524

2/ Sigma Cat. #G-2519

Table 27

Tomato Root Medium

Murashige & Skoog salts[1/]		1 liter packet
Murashige & Skoog vitamins[2/]		1 ml
sucrose		30 gm
gar		8 gm
pH		5.8
distilled water	to	2 liters

[1/] Sigma Cat. #M-5524

[2/] Sigma Cat. #G-2519

Table 28

Populus Callus Initiation (PCI) Medium[1/]

nitrate stock 10X		100.0 ml
sulfate stock 10X		100.0 ml
phosphate & halide stock 10X		100.0 ml
iron & EDTA stock 10X		100.0 ml
vitamin stock 50X		20.0 ml
sucrose		20.0 gm
2,4-D[2/]		1.0 mg
agar		8 gm
pH		5.7
distilled water	to	1 liter

1/ The PCI medium ingredients, e.g. nitrate stock, are shown in Table 31 below.

2/ A herbicide, 2,4-dichlorophenoxyacetic acid.

Table 29

Populus Shoot Initiation (PSI) Medium

nitrate stock 10X		100.0 ml
sulfate stock 10X		100.0 ml
phosphate & halide stock 10X		100.0 ml
iron & EDTA stock 10X		100.0 ml
vitamin stock 50X		20.0 ml
sucrose		20.0 gm
zeatin		0.5 mg
agar		8 gm
carbenicillin		500μg/ml
kanamycin		50μg/ml
pH		5.7
distilled water	to	1 liter

Table 30

Populus Root Medium

nitrate stock 10X		100.0 ml
sulfate stock 10X		100.0 ml
phosphate & halide stock 10X		100.0 ml
iron & EDTA stock 10X		100.0 ml
vitamin stock 50X		20.0 ml
sucrose		20.0 gm
agar		8 gm
pH		5.7
distilled water	to	2 liters

Table 31

Populus media ingredients

Nitrate Stock (10X)

	mg/L
NH_4NO_4	16,500.0 mg
$Ca(NO_3)_2 \cdot 4H_2O$	20,000.0 mg
distilled water	to 1 liter

Sulfate Stock (10X)

	mg/L
$MgSO_4 \cdot 7H_2O$	3,700.0 mg
K_2SO_4	9,900.0 mg
$CuSO_4 \cdot 5H_2O$	2.5 mg (25ml stock sol.)
$ZnSO_4 \cdot 7H_2O$	86.0 mg
$MnSO_4 \cdot H_2O$	151.0 mg
distilled water	to 1 liter

Phosphate and Halide (10X)

	mg/l
KH_2PO_4	1,700.0 mg
$CaCl_2 \cdot 2H_2O$	960.0 mg
H_3BO_3	62.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg (2.5 ml stock sol.)
distilled water	to 1 liter

Iron and EDTA (10X)

	mg/L
$FeSO_4 \cdot 7H_2O$	278.0 mg
Na_2-EDTA$\cdot 2H_2O$	372.0 mg
distilled water	to 1 liter

Vitamin Stock (50X)		mg/200ml
myo-inositol		1,000.0 mg
nicotinic acid		10.0 mg
pyridoxine HCl		10.0 mg
thiamine HCl		10.0 mg
casein hydrolysate		5,000.0 mg
distilled water	to	200 ml

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) NUMBER OF SEQUENCES: 16

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corneyobacter matruchotii
(B) STRAIN: None
(C) INDIVIDUAL ISOLATE: None
(D) DEVELOPMENTAL STAGE: None
(E) HAPLOTYPE: None
(F) TISSUE TYPE: None
(G) CELL TYPE: None

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: None
(B) CLONE: HA nucleating protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Gln Phe Ile Thr Asp Leu Ile Lys Lys Ala Val Asp Phe Phe Lys
1 5 10 15

Gly Leu Phe Gly Asn Lys Gln
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corneyobacter matruchotii
(D) DEVELOPMENTAL STAGE: Stationary Phase Culture
(F) TISSUE TYPE: Bacteria
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 75

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGCAGTTCA TCACTGATCT GATCAAAAAG GCGGTTGATT TTTTTAAAGG TCTGTTCGGT
60
AACAAACAGT AATAG
75

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 260 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Liver
(G) CELL TYPE: Hepatocyte
(H) CELL LINE: None

(vii) IMMEDIATE SOURCE:
(B) CLONE: CAII

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 260

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser His His Trp Gly Tyr Gly Lys His Asn Gly Pro Glu His Trp
1 5 10 15

His Lys Asp Phe Pro Ile Ala Lys Gly Glu Arg Gln Ser Pro Val Asp
20 25 30

Ile Asp Thr His Thr Ala Lys Tyr Asp Pro Ser Leu Lys Pro Leu Ser
35 40 45

Val Ser Tyr Asp Gln Ala Thr Ser Leu Arg Ile Leu Asn Asn Gly His
50 55 60

Ala Phe Asn Val Glu Phe Asp Asp Ser Gln Asp Lys Ala Val Leu Lys
65 70 75 80

Gly Gly Pro Leu Asp Gly Thr Tyr Arg Leu Ile Gln Phe His Phe His
85 90 95

Trp Gly Ser Leu Asp Gly Gln Gly Ser Glu His Thr Val Asp Lys Lys
100 105 110

Lys Tyr Ala Ala Glu Leu His Leu Val His Trp Asn Thr Lys Tyr Gly
115 120 125

Asp Phe Gly Lys Ala Val Gln Glu Pro Asp Gly Leu Ala Val Leu Gly
130 135 140

Ile Phe Leu Lys Val Gly Ser Ala Lys Pro Gly Leu Gln Lys Val Val
145 150 155 160

Asp Val Leu Asp Ser Ile Lys Thr Lys Gly Lys Ser Ala Asp Phe Thr
165 170 175

Asn Phe Asp Pro Arg Gly Leu Leu Pro Glu Ser Leu Asp Tyr Trp Thr
180 185 190

Tyr Pro Gly Ser Leu Thr Thr Pro Pro Leu Leu Glu Cys Val Thr Trp
195 200 205

Ile Val Leu Lys Glu Pro Ile Ser Val Ser Ser Glu Gln Val Leu Lys
210 215 220

Phe Arg Lys Leu Asn Phe Asn Gly Glu Gly Glu Pro Glu Glu Leu Met
225 230 235 240

Val Asp Asn Trp Arg Pro Ala Gln Pro Leu Lys Asn Arg Gln Ile Lys
245 250 255

Ala Ser Phe Lys
260

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Liver
(G) CELL TYPE: Hepatocyte
(H) CELL LINE: None

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 783

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGTCCCATC ACTGGGGGTA CGGCAAACAC AACGGACCTG AGCACTGGCA TAAGGACTTC
60

CCCATTGCCA AGGGAGAGCG CCAGTCCCCT GTTGACATCG ACACTCATAC AGCCAAGTAT
120

GACCCTTCCC TGAAGCCCCT GTCTGTTTCC TATGATCAAG CAACTTCCCT GAGGATCCTC
180

AACAATGGTC ATGCTTTCAA CGTGGAGTTT GATGACTCTC AGGACAAAGC AGTGCTCAAG
240

GGAGGACCCC TGGATGGCAC TTACAGATTG ATTCAGTTTC ACTTTCACTG GGGTTCACTT
300

GATGGACAAG GTTCAGAGCA TACTGTGGAT AAAAAGAAAT ATGCTGCAGA ACTTCACTTG
360

GTTCACTGGA ACACCAAATA TGGGGATTTT GGGAAAGCTG TGCAGGAACC TGATGGACTG
420

GCCGTTCTAG GTATTTTTTT GAAGGTTGGC AGCGCTAAAC CGGGCCTTCA GAAAGTTGTT
480

GATGTGCTGG ATTCCATTAA AACAAAGGGC AAGAGTGCTG ACTTCACTAA CTTCGATCCT
540

CGTGGCCTCC TTCCTGAATC CCTGGATTAC TGGACCTACC CAGGCTCACT GACCACCCCT
600

CCTCTTCTGG AATGTGTGAC CTGGATTGTG CTCAAGGAAC CCATCAGCGT CAGCAGCGAG
660

CAGGTGTTGA AATTCCGTAA ACTTAACTTC AATGGGGAGG GTGAACCCGA AGAACTGATG
720

GTGGACAACT GGCGCCCAGC TCAGCCACTG AAGAACAGGC AAATCAAAGC TTCCTTCAAA
780

TAA
783

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Intestinal epithelial lining
(G) CELL TYPE: Epithelial cell

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Lys Ser Pro Glu Glu Leu Lys Gly Ile Phe Glu Lys Tyr Ala Ala
1 5 10 15

Lys Glu Gly Asp Pro Asn Gln Leu Ser Lys Glu Glu Leu Lys Leu Leu
20 25 30

Leu Gln Thr Glu Phe Pro Ser Leu Leu Lys Gly Pro Ser Thr Leu Asp
35 40 45

Glu Leu Phe Glu Glu Leu Asp Lys Asn Gly Asp Gly Glu Val Ser Phe
50 55 60

Glu Glu Phe Gln Val Leu Val Lys Lys Ile Ser Gln
65 70 75

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 234 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Gene Patterned after Bovine Calcium Binding Protein"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Intestinal epithelial lining
(G) CELL TYPE: Mucosa
(H) CELL LINE: None

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 234

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGAAATCTC CGGAAGAACT GAAAGGTATC TTTGAAAAAT ACGCGGCGAA AGAAGGTGAT 60

CCGAACCAGC TGTCTAAAGA AGAACTGAAA CTGCTGCTGC AGACTGAGTT CCCGTCTCTG 120

CTGAAAGGTC CGTCTACTCT GGATGAACTG TTCGAAGAGC TCGATAAAAA CGGTGATGGT
180

GAAGTTTCTT TCGAAGAGTT CCAGGTTCTG GTTAAAAAGA TCTCTCAGTA ATAG
234

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Liver
(G) CELL TYPE: Hepatocyte
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Lys Ser Cys Cys Ser Cys Cys Pro Met Ser Cys Ala Lys Cys Ala

Gln Gly Cys Ile Cys Lys Gly Ala Ser Glu Lys Cys Ser Cys Cys Ala
20 25 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Sequence developed from Human Liver Metallothionein"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapien

(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Liver
(G) CELL TYPE: Hepatocyte
(H) CELL LINE: None

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 102

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGAAGAGCT GCTGCTCCTG CTGCCCCATG AGCTGTGCCA AGTGTGCCCA GGGCTGCATA
60

TGCAAAGGGG CATCAGAGAA GTGCAGCTGC TGTGCCTAAT AG
102

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR Forward Primer for Carbonic Anhydrase II"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 42

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGATCCTCTA GAATGTCCCA TCACTGGGGG TACGGCAAAC AC
42

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Reverse PCR Primer for Carbonic Anhydrase II"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCGGGGAGC TCTTATTTGA AGGAAGCTTT GATTTGCCTG
40

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Hind III-Sal I Cassette for gene combinations"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGGTCGA CAGCT
55

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Hind III - Hpa I Cassette for Gene Combinations"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGGTTAA CAGCT
55

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Hind III - Kpn I Cassette for gene combinations"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 56

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGGGTAC CAAGCT
56

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "EcoR I - Sal I cassette for gene combinations"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant

(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 57

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGAGTCG ACGAATT
57

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "EcoRI - Hpa Cassette for Gene Combinations"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant
(C) UNITS: 57

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGAGTTA ACGAATT 57

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "EcoR I - Kpn I Cassette for Gene Combinations"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not Relevant
(D) DEVELOPMENTAL STAGE: Not Relevant
(F) TISSUE TYPE: Not Relevant
(G) CELL TYPE: Not Relevant
(H) CELL LINE: Not Relevant

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Not Relevant
(B) MAP POSITION: Not Relevant

(C) UNITS: 57

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTACATATTG TCGTTAGAAC GCGTAATACG ACTCACTATA GGGAGAGGTA CCGAATT
57

WHAT IS CLAIMED IS:

1. A process of enhancing plant growth, comprising the steps of:

selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein, or biomineralization protein, and a promoter operatively located upstream of said heterologous gene; and

growing said plant in a growth medium.

2. A process according to claim 1, wherein said plant is selected from the group consisting of bean, cabbage, carrot, corn, cotton, eggplant, guayule, pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato, trees and wheat.

3. A process according to claim 1, wherein said heterologous gene is an animal gene.

4. A process according to claim 3, wherein said heterologous gene is a mammalian gene.

5. A process according to claim 1, wherein said heterologous gene is a bacterial gene.

6. A process according to claim 1, wherein said heterologous gene is a yeast gene.

7. A process according to claim 1, wherein said heterologous gene is a plant gene.

8. A process according to claim 1, wherein said heterologous gene is a synthetic gene.

9. A process of enhancing plant growth, comprising the steps of:

selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one carbonic anhydrase, and a promoter operatively located upstream of said heterologous gene; and

growing said plant in a growth medium.

10. A process according to claim 9, wherein said heterologous gene is an animal gene.

11. A process according to claim 9, wherein said heterologous gene is a mammalian gene.

12. A process according to claim 9, further comprising the step of adding a source of calcium to said growth medium.

13. A process of enhancing plant growth, comprising the steps of:

selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one calcium binding protein, and a promoter operatively located upstream of said heterologous gene; and

growing said plant in a growth medium.

14. A process according to claim 13, wherein said calcium binding protein is selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin, calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain, calreticulin, calretinin, calsequestrin, caltractin,

gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin.

15. A process according to claim 14, wherein said heterologous gene is selected from the group consisting of bovine, human, mouse and rat calcium binding protein.

16. A process according to claim 13, further comprising the step of adding a source of calcium to said growth medium.

17. A process of enhancing plant growth, comprising the steps of:

selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one metal binding protein, and a promoter operatively located upstream of said heterologous gene; and

growing said plant in a growth medium.

18. A process according to claim 17, wherein said metal binding protein is selected from the group consisting of metallothionein, ubiquitin, zinc binding protein, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5 and HIV-1 enhancer-binding protein.

19. A process of enhancing plant growth, comprising the steps of:

selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one biomineralization protein,

and a promoter operatively located upstream of said heterologous gene; and

growing said plant in a growth medium.

20. A process according to claim 19, wherein said biomineralization protein is a hydroxyapatite nucleating protein.

21. A process according to claim 20, wherein said hydroxyapatite nucleating protein comprises:

(i) at least two lysines, which comprise a phosphate binding site, wherein said lysines are at a distance of 10 Angstroms or less from each other;

(ii) at least two aspartic acids, which comprise a calcium ion binding site, wherein said aspartic acids are in a trough;

(iii) an alpha helical structure in a portion of the protein; and

(iv) a two amino acid distance between the phosphate binding site of (i) and the calcium ion binding site of (ii).

22. A process according to claim 21, wherein said hydroxyapatite nucleating protein has at least two phosphate binding sites each containing two lysines at a distance of 10 Angstroms or less from each other.

23. A process according to claim 20, wherein said hydroxyapatite nucleating protein comprises the amino acid sequence identified as SEQ ID NO: 1.

24. A process according to claim 20, wherein said heterologous gene encodes for the hydroxyapatite nucleating protein that comprises the amino acid sequence identified as SEQ ID NO: 1.

25. A process according to claim 24, wherein said heterologous gene encoding for said hydroxyapatite nucleating protein comprises the nucleotide sequence identified as SEQ ID NO: 2.

26. A plant comprising at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one of carbonic anhydrase, calcium binding protein, metal binding protein, or biomineralization protein, and a promoter operatively located upstream of said heterologous gene.

27. A plant according to claim 26, wherein said plant is selected from the group consisting of bean, cabbage, carrot, corn, cotton, eggplant, guayule, pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato, trees and wheat.

28. A plant according to claim 26, wherein said heterologous gene is an animal gene.

29. A plant according to claim 28, wherein said heterologous gene is a mammalian gene.

30. A plant according to claim 26, wherein said heterologous gene is a bacterial gene.

31. A plant according to claim 26, wherein said heterologous gene is a yeast gene.

32. A plant according to claim 26, wherein said heterologous gene is a plant gene.

33. A plant according to claim 26, wherein said heterologous gene is a synthetic gene.

34. A plant comprising at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one carbonic anhydrase, and a promoter operatively located upstream of said heterologous gene.

35. A plant according to claim 34, wherein said heterologous gene is an animal gene.

36. A plant according to claim 35, wherein said heterologous gene is a mammalian gene.

37. A plant comprising at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one calcium binding protein, and a promoter operatively located upstream of said heterologous gene.

38. A plant according to claim 37, wherein said calcium binding protein is selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin, calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain, calreticulin, calretinin, calsequestrin, caltractin, gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin.

39. A plant according to claim 37, wherein said heterologous gene is selected from the group consisting of bovine, human, mouse and rat calcium binding protein.

40. A plant comprising at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least

one metal binding protein, and a promoter operatively located upstream of said heterologous gene.

41. A plant according to claim 40, wherein said metal binding protein is selected from the group consisting of: metallothionein, ubiquitin, zinc binding protein, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5 and HIV-1 enhancer-binding protein.

42. A plant comprising at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one biomineralization protein, and a promoter operatively located upstream of said heterologous gene.

43. A plant according to claim 42, wherein said biomineralization protein is a hydroxyapatite nucleating protein.

44. A plant according to claim 43, wherein said hydroxyapatite nucleating protein comprises:

(i) at least two lysines, which comprise a phosphate binding site, wherein said lysines are at a distance of 10 Angstroms or less from each other;

(ii) at least two aspartic acids, which comprise a calcium ion binding site, wherein said aspartic acids are in a trough;

(iii) an alpha helical structure in a portion of the protein; and

(iv) a two amino acid distance between the phosphate binding site of (i) and the calcium ion binding site of (ii).

45. A plant according to claim 44, wherein said hydroxyapatite nucleating protein has at least two

phosphate binding sites each containing two lysines at a distance of 10 Angstroms or less from each other.

46. A plant according to claim 43, wherein said hydroxyapatite nucleating protein comprises the amino acid sequence identified as SEQ ID NO: 1.

47. A plant according to claim 43, wherein said heterologous gene encodes for the hydroxyapatite nucleating protein that comprises the amino acid sequence identified as SEQ ID NO: 1.

48. A process according to claim 47, wherein said heterologous gene encoding for said hydroxyapatite nucleating protein comprises the nucleotide sequence identified as SEQ ID NO: 2.

49. Seeds obtained from the plant according to claim 26.

50. Seeds obtained from the plant according to claim 28.

51. Seeds obtained from the plant according to claim 34.

52. Seeds obtained from the plant according to claim 35.

53. Seeds obtained from the plant according to claim 37.

54. Seeds obtained from the plant according to claim 38.

55. Seeds obtained from the plant according to claim 40.

56. Seeds obtained from the plant according to claim 41.

57. Seeds obtained from the plant according to claim 42.

58. Seeds obtained from the plant according to claim 43.

59. Seeds obtained from the plant according to claim 44.

60. Seeds obtained from the plant according to claim 45.

61. A process of enhancing calcium accumulation in a plant comprising the steps of:

selecting a plant having at least one heterologous DNA construct, said heterologous DNA construct comprising at least one heterologous gene encoding for at least one of calcium binding protein, metal binding protein, or biomineralization protein, and a plant promoter operatively located upstream of said heterologous gene; and

growing said plant in a growth medium.

62. A process according to claim 61, wherein said heterologous DNA construct comprises at least one heterologous gene encoding for at least one calcium binding protein.

63. A process according to claim 62, wherein said calcium binding protein is selected from the group consisting of aequorin, annexin, cadherin, calbindin, calcineurin, calcitonin, calcium transporting ATPase, calcyclin, calcyphosin, calcyphosine, caldesmon, calgizzarin, calmodulin, calnexin, calpain,

calreticulin, calretinin, calsequestrin, caltractin, gelsolin, hydroxyapatite nucleating protein, osteonectin, osteopontin, S 100, severin, transcalcin, troponin, tubulin and villin.

64. A process according to claim 61, wherein said heterologous DNA construct comprises at least one heterologous gene encoding for at least one metal binding protein.

65. A process according to claim 64, wherein said metal binding protein is selected from the group consisting of metallothionein, ubiquitin, zinc binding protein, S-adenosyl homocysteine hydrolase, peptidylglycine alpha amidating monoxygenase 5 and HIV-1 enhancer-binding protein.

66. A process according to claim 61, wherein said heterologous DNA construct comprises at least one heterologous gene encoding for at least one biomineralization protein.

67. A process according to claim 66, wherein said biomineralization protein is a hydroxyapatite nucleating protein.

68. A process according to claim 67, wherein said hydroxyapatite nucleating protein comprises:

(i) at least two lysines, which comprise a phosphate binding site, wherein said lysines are at a distance of 10 Angstroms or less from each other;

(ii) at least two aspartic acids, which comprise a calcium ion binding site, wherein said aspartic acids are in a trough;

(iii) an alpha helical structure in a portion of the protein; and

(iv) a two amino acid distance between the phosphate binding site of (i) and the calcium ion binding site of (ii).

69. A process according to claim 68, wherein said hydroxyapatite nucleating protein has at least two phosphate binding sites each containing two lysines at a distance of 10 Angstroms or less from each other.

70. A process according to claim 67, wherein said hydroxyapatite nucleating protein comprises the amino acid sequence identified as SEQ ID NO: 1.

71. A process according to claim 67, wherein said heterologous gene encodes for the hydroxyapatite nucleating protein that comprises the amino acid sequence identified as SEQ ID NO: 1.

72. A process according to claim 71, wherein said heterologous gene encoding for said hydroxyapatite nucleating protein comprises the nucleotide sequence identified as SEQ ID NO: 2.

73. A process according to claim 61, wherein said plant is selected from the group consisting of bean, cabbage, carrot, corn, cotton, eggplant, guayule, pea, peanut, potato, pumpkin, rice, rye, soybean, squash, sugarcane, sunflower, tobacco, tomato, trees and wheat.

74. A process according to claim 61, wherein said heterologous gene is an animal gene.

75. A process according to claim 61, wherein said heterologous gene is a bacterial gene.

76. A process according to claim 61, wherein said heterologous gene is a plant gene.

77. A process according to claim 61, wherein said heterologous gene is a synthetic gene.

78. A process according to claim 61, further comprising the step of adding a source of calcium to said growth medium.